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VOLUME 21 NUMBER 2

PLANT PHYSIOLOGY

APRIL, 1946

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PUBLISHED QUARTERLY
BY
THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS
AT N. QUEEN ST. AND McGOVERN AVE., LANCASTER, PA.
THE SCIENCE PRESS PRINTING COMPANY

Entered as second-class matter March 25, 1926, at the post office at Lancaster, Pa., under the Act of August 24, 1912.
Accepted for mailing at the special rate of postage provided for in paragraph 4, section 538. Authorized April 9, 1926.

PLANT PHYSIOLOGY

A QUARTERLY JOURNAL
DEVOTED TO ALL PHASES OF PLANT PHYSIOLOGY

Established 1926

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Subscription price, $3.00 a year. Single copies, $2.50 post free. Members of the American Society of Plant Physiologists receive the journal in connection with dues paid to the Society.

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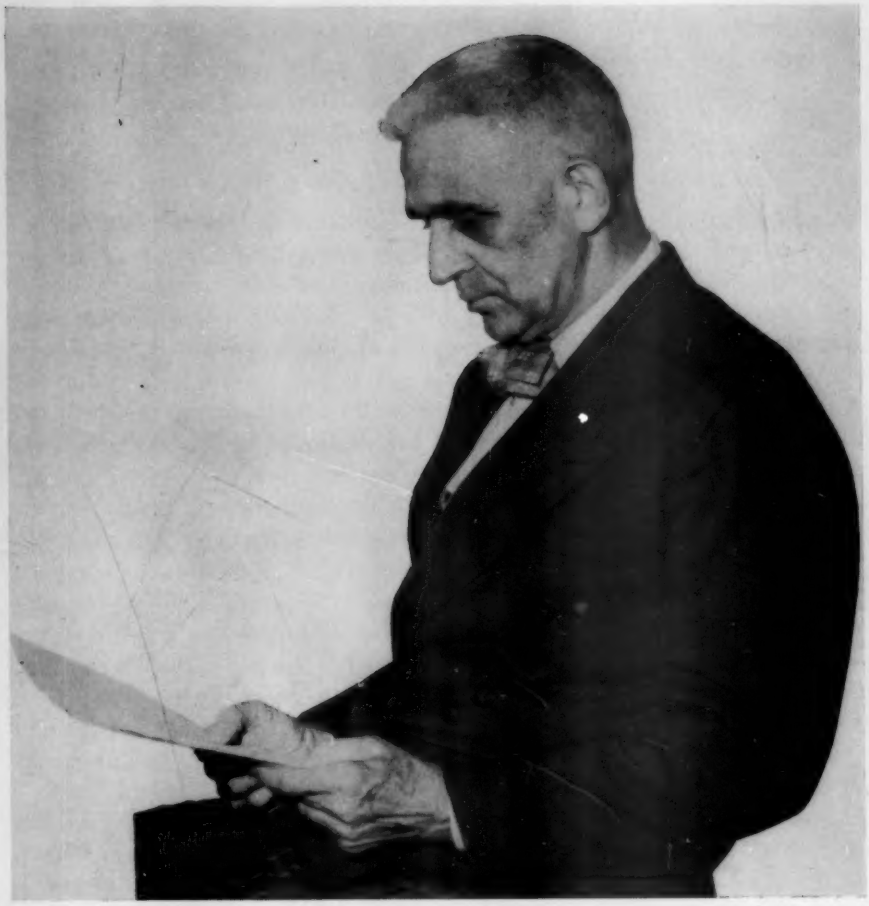


EDWIN CYRUS MILLER

Vol. 21 No. 2

PLANT PHYSIOLOGY

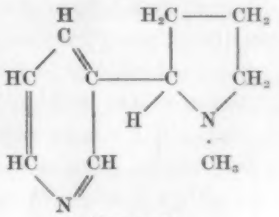
APRIL, 1946

DEVELOPMENT OF SOME RECENT CONCEPTS IN THE PHYSIOLOGICAL CHEMISTRY OF THE TOBACCO ALKALOIDS[1]

RAY F. DAWSON

The plant alkaloids constitute a group of nitrogenous substances which possess great interest for the organic chemist because of their intricate molecular structure and for the pharmacologist because of the extraordinary physiological effects that many of them exert when introduced into the animal body. It is a regrettable fact, however, that so little is known of their physiological functions and of the mechanisms by which they are synthesized within the plant cell. Concerning these problems, the lack of an obvious experimental approach has led to extensive speculation. For instance, PICTET, who first synthesized nicotine, assumed that the alkaloids may represent degradation products of proteins, nucleic acids, and chlorophyll (**35**). The great JUSTUS VON LIEBIG implied their use as substitutes for the mineral bases within the plant body when he developed his "mineral theory" (**3, 26**). The most prevalent view at the present time has been well expressed by CROMWELL (**5**) and by VICKERY (**42**). This hypothesis holds that the alkaloids represent by-products of nitrogen metabolism in which irreversible reactions are involved and that they differ from the end-products of nitrogen metabolism in animals by their retention within the body of the organism.

Within the past decade a serious attempt has been made to ascertain the nature of nicotine production in the tobacco plant. Several factors have contributed to this choice, but the most important have undoubtedly been the



NICOTINE

worldwide use of nicotine as a mild narcotic and the relative ease with which the alkaloid may be quantitatively assayed. From these investigations cer-

[1] The ninth STEPHEN HALES Address. 1945.

tain well-defined concepts have been developed concerning 1) the locus of nicotine synthesis, 2) the extent of translocation, and 3) the nature of nicotine accumulation in the growing plant body (**9, 14, 19, 32**). It has seemed worthwhile to retrace here the development of these concepts in order to call attention to the important new opportunities which they provide for basic research in the physiological chemistry of nicotine in particular and of the plant alkaloids in general.

Distribution of nicotine in the growing plant

The very excellent work of VICKERY and his collaborators (**43, 45**) has provided a picture of the accumulation of nicotine in the leaf and stem of the tobacco plant during its growth and developmental stages. The general pattern of accumulation begins with the seedling, nine to eleven days after germination, for the mature tobacco seed does not contain detectable quantities of the alkaloid. When the young plants have attained transplanting size, the differential nature of alkaloid distribution within the plant body has already become apparent. That is, nicotine is present in appreciable quantities in both root and stem, but by far the greatest proportion resides in the leaves. Furthermore, the proportion of the total nicotine of the plant to be found in the leaves throughout most of the period of growth (**45**) remains within the extremely limited range of 85 to 88 per cent. As VICKERY has pointed out, "The constancy of the proportion in the leaves is remarkable in view of the rapidly changing relative proportions of leaf and stem tissue as the plant grows, and raises an interesting question with regard to the kind of cells that are capable of synthesizing nicotine." It is also of interest to note that the most rapid rate of overall nicotine accumulation may occur at a time in the life of the plant when growth in terms of dry weight increase has all but ceased (**45**). Clearly, therefore, nicotine accumulation, in the aerial organs at least, is not directly associated with the growth processes of the tobacco plant. This conclusion has, indeed, already been expressed by MOTHES (**31**). As VICKERY has suggested, the interpretation of these and of other aspects of nicotine accumulation in tobacco, particularly the quantitative relationship between nicotine nitrogen and total nitrogen in the leaf, is difficult "in view of our ignorance of the exact position of nicotine in the scheme of nitrogen metabolism in the tobacco plant."

Certainly, one of the important contributions of VICKERY and his associates lies in their emphasis upon the lack of information concerning the "kind of cells," that is, the exact locus or loci within the plant body where nicotine formation can occur. Hence it becomes apparent that the dilemma of interpretation can be dispelled only by going back to some more fundamental problems among which is the ultimate origin of nicotine.

The locus of nicotine formation

The first extensive physiological investigation of nicotine formation in tobacco was carried out by MOTHES (**31**). Working within the framework

of older suppositions concerning the *in situ* nature of alkaloid synthesis and the doctrine of phloem-transport of organic compounds, MOTHES attempted to alter or to block nicotine formation by manipulations of the environment of the plant. He found the process of nicotine accumulation to be an extremely stubborn object for experimentation. So long as the leaf was attached to the growing plant, it continued to acquire nicotine even under such drastic conditions as nitrogen deprivation and carbohydrate starvation. No appreciable transport of alkaloid from the leaf could be detected. It seems reasonable to conclude, therefore, that the nicotine metabolism of the tobacco leaf is characterized by a ponderous stability and that the usual methods of physiological experimentation can not be expected to yield information of much value.

The methodology of experimental morphology would seem to offer more promise. It has been noted above that as long as the tobacco leaf is attached to the growing plant, nicotine accumulates regardless of environmental circumstances. MOTHES observed, however, that excised leaves ceased immediately to acquire the alkaloid in spite of the nature of their subsequent treatment. This phenomenon of accumulation interrupted by excision has, of course, long been known to occur in tobacco leaves which are stripped from the plant preparatory to processing for commercial use. The work of MOTHES and the researches of VICKERY and PUCHER (**44, 46**) on the chemical changes that occur in excised leaves during culture under a wide variety of conditions have, nevertheless, brought into bold relief the simple fact that organic connection between leaf and plant is necessary to continued increase in the nicotine content of the leaf.

In logical continuation of this observation, the following points have been experimentally established. First, when tobacco shoots are excised and cultured in water, they, likewise, cease at once to accumulate nicotine (**7**). Secondly, if segments of tobacco stems are excised and placed in moist chambers, they send out lateral branches which are nicotine free (**10**). Thirdly, excised segments of tobacco stems placed in moist chambers also produce callous tissue at their basal surfaces. This callous tissue contains no alkaloid (**10**). Fourthly, the callous tissue that develops on the cut ends of the petioles of excised leaves held in moist chambers contains nicotine, but this nicotine is transported from the leaf blade and hence does not lead to an increase in the total amount of alkaloid in the leaf (unpublished data). Finally, nicotine does accumulate in extraordinarily large amounts in excised leaves that have been rooted in sand (**9**). As a result of these experiments, it is possible to conclude that not only is an organic connection between leaf and plant necessary for continued alkaloid accumulation, but also the plants (or the leaf) must bear roots. Consequently, it becomes necessary to look to the root for the source of nicotine or of its precursor(s).

NATH in India (**33**) and BERNARDINI in Italy (**1**) were among the first to report the remarkable results to be obtained by grafting tobacco upon tomato. These authors observed a great decrease in the concentration of

nicotine in the leaves of the tobacco scions. NATH reported the equally interesting observation that the reciprocal graft (i.e., tomato scion on tobacco stock) accumulated nicotine in both stock and scion. A year later HASEGAWA (**18**) confirmed these results. Subsequently, EVTUSHENKO (**15**), KUSMENKO and TIKHVINSKAYA (**25**), SHMUCK (**37**), SHMUCK, SMIRNOV and ILYIN (**39**), and SHMUCK, KOSTOFF, and BOROZDINA (**38**) brought forth extensive confirmation based upon grafts of tobacco with tomato and with a variety of other species. The experimental data collected by these investigators, fortunately, were characterized by more agreement than were the interpretations applied to them. The earlier report by NATH and the observations of BERNARDINI and HASEGAWA were based upon data too meager to permit more than speculation concerning their significance. It is difficult to understand, however, why the far more extensive and well planned work of SHMUCK and his colleagues was regarded by them to support the mystical notion of KRENKE (**24**) concerning the release of "hidden properties" within the scions under the influence of "new developmental conditions." SHMUCK definitely discounted the possibility of translocation of the alkaloid from root to shoot (**38, 39**).

More recent investigations (**10**) have considerably simplified the problem of interpretation. Tobacco stocks and scions were defoliated and cut as short as possible in order to reduce materially their initial content of nicotine. They were then reciprocally grafted with tomato. The tobacco leaves that subsequently developed upon tomato stocks were nicotine-free with the exception of the lowermost, which contained traces of the alkaloid. From the upper stem and the inflorescence of the tobacco scion there was isolated a base which could be determined as "nicotine" by the usual silicotungstic acid precipitation but which was not identical with this alkaloid. On the other hand, nicotine did accumulate extensively in tomato scions grown upon tobacco stocks. Such accumulation was greatest in the lower and older leaves, and extensive injury occurred to the tissues of these leaves. One of the most interesting observations, however, in connection with the problem of the origin of nicotine is found in the sectoral nature of nicotine accumulation in the tomato component of approach grafts of tobacco with tomato (**10**). Such a response could indicate only an upward transport of nicotine through the xylem. This view was confirmed by the isolation of nicotine from the sap that bleeds from the cut stumps of decapitated tobacco plants. The latter observation, likewise, demonstrated that it is nicotine itself that is transported to the leaf and not some precursor which can be converted to nicotine once it has reached the leaf cells. These findings have since been confirmed completely by the work of MOTHES and HIEKE (**32**) and in large part by the subsequent report of PAL and NATH (**34**).

To recapitulate, the evidence obtained from excised organs and from reciprocal grafts with non-alkaloid producing species has shown that nicotine is not produced *in situ* in the leaf and stem of the tobacco plant but rather is translocated to these organs there to accumulate.

The next question, of course, concerns the actual locus of the nicotine synthetic mechanism within the plant body. The absence of nicotine in tobacco stems and leaves when grafted close to the roots of tomato stocks obviously suggests that the alkaloid is formed only in the roots of the tobacco plant. Final proof for the correctness of this view was obtained when nicotine was isolated from both the tissues and the spent culture fluids of excised tobacco roots in sterile culture (**11**).

Many heretofore unexplained characteristics of nicotine content in tobacco now seem susceptible of interpretation. Among these may be mentioned the differential nature of alkaloid accumulation in leaves and stems during growth (**45**); the extensive enrichment of the leaves of topped tobacco plants with respect to nicotine; the low nicotine content of the rapidly grown sucker or ratoon crop (**30**); the abrupt termination of alkaloid accumulation in excised tobacco leaves and shoots; and the difficulty encountered by MOTHES in his experiments with respect to altering the nicotine content of the leaves of intact plants by manipulating those environmental factors which influence principally the aerial shoot.

The extent and nature of nicotine transport

Evidence was presented in the preceding section to show that nicotine is translocated from root to shoot and that the pathway for at least the bulk of such transport is undoubtedly the xylem. It would seem reasonable to assume, therefore, that an examination of the distribution of the alkaloid in various parts of the stem and in leaves at different levels on the stem might reveal the major currents of such transport. MOTHES (**31**) reported, and indeed general experience shows, that in the absence of senescent changes the total nicotine content per leaf decreases with increase in height of the leaf position on the stalk. That is, so long as they remain anabolically active, the older leaves contain the greatest quantities of alkaloid. The distribution of nicotine in the stems has also been examined (**9**). In this case, the greatest accumulation of the alkaloid is found in the cortex, although the pith and xylem contain appreciable amounts. Taking into consideration the additional fact that by far the greater proportion of the total nicotine of the aerial shoot is located in the leaves, it at once becomes apparent that the patterns of nicotine distribution within the shoot are identical, at least qualitatively, with what might be expected if the alkaloid were translocated in the transpiration stream. To state the matter in another way, the alkaloid can be considered to accumulate in the different regions of the shoot in proportion to the anticipated temporal duration and relative intensity of their transpirational losses. Based upon this viewpoint, a flow-diagram of nicotine transport and deposition within the plant body may be constructed with interesting results. It may be noted that such a diagram predicts the differential character of nicotine distribution in the tobacco leaf as determined experimentally by CICERONE and MAROCCHI (**4**).

At this point it seems desirable to raise the question of the state in which

nicotine occurs in the cells and cell fluids. In the above discussion, it has been assumed that the sole forces governing nicotine distribution within the plant body are diffusion and transpiration. If, however, nicotine were to associate electively in salt formation with any particular acidic substance or substances and this were to be followed by the appearance of a solid phase, then it might be expected that the foregoing interpretation of the nature of nicotine accumulation would be considerably in error. Almost without exception, texts and reference books contain the statement that nicotine is found in tobacco leaves as the salt of malic and citric acids. Now, the alkaloid is a relatively feeble di-acid base (**20**) and would be expected to undergo salt formation readily. In the very low concentration in which it occurs in the leaf tissue fluids (0.012 molar or approximately 0.20 per cent. in the experiments of VICKERY and PUCHER (**45**) at the 75-day collection), it is not likely that precipitation of nicotine as the salt of an organic acid would take place. On the contrary, since most of the salts of nicotine are readily soluble in water, it is far more likely that the alkaloid exists in the living cell in a complex series of equilibria with negatively charged particles any and all of which may undergo extensive changes in relative concentration with drift in time and in metabolic activity. Therefore, nicotine cannot be said to associate electively with any one or two acids but must be thought of as entering into the general buffering system of the plant cell. Indeed, to the limited extent that its low concentration in terms of normality in the cell sap permits, it may be said that its chemical properties demand recognition of such a function for this alkaloid in tobacco physiology.

The nature of the nicotine synthetic process

As an approach to the highly interesting but largely undocumented subject of the intermediary metabolism of nicotine, it is necessary to observe that the localization of the synthetic mechanism with respect to specific tissues or developmental zones within the tobacco root has not yet been investigated. Likewise, information concerning the nature of the environmental factors which can influence the rate of synthesis is meager and often conflicting. It seems fairly certain, however, that the extent of accumulation in the leaf and presumably, therefore, the overall rate of production by the root can be increased by growing the plants in a heavy soil as contrasted with a light soil (private communication), in a dry soil as compared with a moist soil (**6**), and by topping and suckering the plants during the growth period (**30**). Interpretation of these results is greatly complicated by the lack of suitable data on concomitant shifts in root-shoot ratio. The same may be said of the results obtained from the application of nitrogenous fertilizers (**31**).

Evidence has been obtained (**45**) which indicates that nicotine disappears as such during the profound redistribution of metabolites that occurs within the plant body following the onset of sexual reproduction. In the last stages of growth, therefore, the rate of nicotine accumulation in the

plant as a whole is determined by the progressive difference between the rate of synthesis and the rate of utilization or breakdown. This differential is probably augmented by a simultaneous diversion of the carbohydrate output of the leaves from root to inflorescence. Indeed, many observations (unpublished) point to the necessity of continued and adequate supplies of carbohydrates in maintaining maximum rates of nicotine output by the roots. In this connection, the remarkable changes that take place as a result of topping tobacco plants during the growth period are of interest. In such cases, there are no reproductive structures or fruits to monopolize the food supply. The result is that the leaves become greatly enlarged due to abnormal increases in the size of the parenchymatous cells (**49**). Associated with this change is usually a very considerable increase in alkaloid concentration (**30**). The magnitude of such increase seems to be greater than would be expected on the basis of an increase in the root-shoot ratio alone.

Aside from circumstantial evidence concerning the necessity of ample supplies of carbohydrate, nothing is known concerning the influence of temperature, oxygen tension, nitrogen supply, and similar variables on the absolute rate of nicotine synthesis in the roots. This field obviously provides much opportunity for future research.

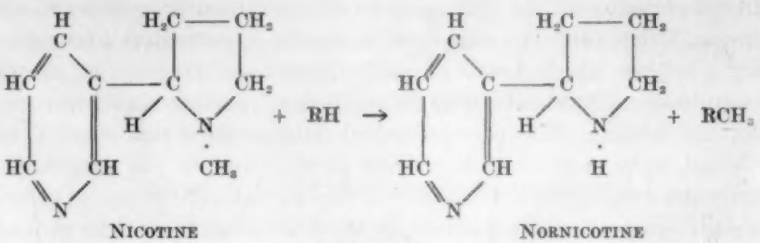
In connection with the identification of possible intermediates in alkaloid synthesis, TRIER (**48**) has suggested a purely hypothetical and rather improbable scheme which would yield the basic ring structure of nicotine by the simultaneous oxidative decarboxylation of proline and nicotinic acid. KLEIN and LINSER (**23**) have published data to show that excised tobacco leaves can make nicotine from proline alone. GORTER (**17**) repeated these experiments and, using a somewhat different method for the expression of data, failed to confirm the results. A third investigation of the problem has also been reported (**7**) in which even larger increases were obtained by feeding nicotinic acid to excised tobacco shoots than were obtained by feeding proline. The recent discovery that nicotine is not normally produced in important amounts in tobacco leaves and the failure of the investigators cited above to establish the validity of analytically determined increases in nicotine content by suitable statistical controls necessitates the adoption of a somewhat skeptical attitude toward such results. Although it seems very unlikely, the possibility cannot be denied that leaves may be found to manufacture at least small amounts of nicotine provided suitable intermediates in the total synthesis are supplied by the investigator and/or by the root of the intact plant. In this connection, a repetition of the experiments described above utilizing naturally nicotine-free tobacco leaves from appropriately grafted plants should clarify once and for all the position of nicotinic acid and proline in the alkaloid metabolism of the aerial organs.

VICKERY (**41**) has attempted to identify the possible precursors of nicotine by another approach. Rather than feed the plant with hypothetical intermediates, this investigator resorted to direct isolation from the seed meal of substances that might play a part in the formation of nicotine during

the germination of the seed. This interesting procedure led to no positive conclusions perhaps due to the lack of suitable methods for the isolation and separation of the rather large proportion of unknown nitrogenous substances that was encountered.

The synthesis of secondary tobacco alkaloids

Nicotine is accompanied in many strains of tobacco by variable amounts of nornicotine. It is interesting to note that nornicotine accumulates most extensively in those varieties of cigarette tobaccos that have been selected for low nicotine content (**28**). That is, there is an intimation that as the nicotine producing capacity of the plant is reduced, its ability to manufacture nornicotine is correspondingly increased. It has recently been shown (**13, 14**) that nornicotine synthesis is, indeed, closely related to that of nicotine insofar as the former alkaloid is produced at the expense of the latter. The overall change involved in the transformation of nicotine into nornicotine is merely the substitution of a hydrogen atom for a methyl group. The simplest assumption to make with regard to the probable mechanism is that nicotine participates in a transmethylation reaction with an enzyme system and a methyl acceptor (Scheme I). In view of the fact that only nicotine



NICOTINE NORNICOTINE

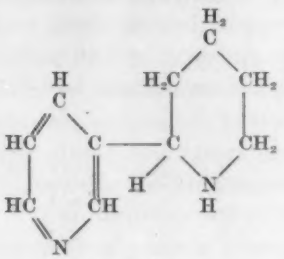
SCHEME I

accumulates in tomato scions when these are grafted to the roots of species that normally contain nornicotine, it has been suggested that the roots of the strains and species in question produce nicotine in the usual manner. This is subsequently transported to the leaves where it is slowly demethylated (**14**). All that is known of the nature of the hypothetical transmethylating system is that it is inheritable and that it possesses a limited working capacity.

Regardless of the lack of detailed information concerning the real nature of the process by which nicotine is changed to nornicotine, it is perfectly clear that nornicotine arises by secondary processes within the leaf. There is as yet no evidence that any organ of the plant can carry out a total synthesis of nornicotine *in situ*, although the possibility that the formation of nicotine in the root may proceed through the reverse process (i.e., the methylation of nornicotine) to that found in the leaf must be investigated.

Many reports may be found in the literature to the effect that F_1 hybrids between *Nicotiana tabacum* and *N. glauca* and scions of *N. glauca* grafted upon *N. tabacum* stocks contain only anabasine in spite of the fact that nico-

tine synthesis in *N. tabacum* is usually far more intensive than is the production of anabasine in *N. glauca* (**40**). If it had been true, this relationship would have intimated, among other possibilities, a competition between the synthetic mechanisms of nicotine and anabasine for a common precursor. The stimulating prospect of discovering such a system has been eliminated by the demonstration (**14**) that anabasine formation does not predominate in the genetical sense over nicotine production in the various combinations



ANABASINE

described above. Rather, the extensive secondary conversion in these combinations of nicotine to nornicotine and the difficulty encountered in identifying the latter substance in the presence of anabasine have contributed to erroneous interpretations. It should be noted that the demonstration of independent anabasine synthesis in both root and shoot of *Nicotiana glauca* (**12**) has shown that the total synthesis of alkaloids is not an inherently unique property of root tissues.

The physiological significance of nicotine

Insofar as it is justifiable to attempt to blueprint the physiology of the tobacco plant and to assign a function to each of the constituents thereof, it would seem well to list the more obvious alternatives with respect to nicotine. For instance it may be possible that nicotine originates as a by-product of a number of irreversible and physiologically useless reactions. Or it may be that it is formed for any one of several reasons as a by-product of chemical reactions that do play a rôle in cell metabolism. Again, the final steps in the synthesis of the alkaloid molecule may involve reactions of use to the cell; or the finished product may itself participate in important activities which are not at present recognized.

It will be noted that the views expressed prior to 1942 were based upon the assumption that nicotine is produced largely in the green leaf and that its physiological import must, therefore, be linked in some way with this organ. From this point of view, the great variation that occurs in nicotine content of different strains and crops of tobacco; the fact that tobacco scions can grow and develop quite normally on tomato stocks without more than minute traces of available nicotine; and the failure of nicotine fed through the cut stems of excised tobacco shoots to alter nitrogen metabolism appreciably (**8**), all support the conclusion that perhaps nicotine plays no important

rôle in the aerial organs. It is obvious, therefore, that an investigation of the effects of nicotine upon the roots of the tobacco plant is necessary. The results of some preliminary experiments, as yet unpublished, are available and are outlined here solely for the purpose of indicating the direction which future investigations of alkaloid physiology are expected to take in this laboratory.

In these experiments, Connecticut Broadleaf tobacco plants were grown in sand culture with a mineral nutrient solution that contained nitrogen only in the form of nitrate for approximately seven weeks. At that time, one-half the plants were given a supplement of 0.10 per cent. nicotine as the hydrochloride. This treatment was maintained for ten days: one-half of the nicotine-fed plants and one-half of the control plants were then harvested. For another nineteen days the remaining plants were watered only with tap water and received no more nutrient solution. These were likewise harvested. The results were rather astonishing.

In the first place, the roots of the plants to which nicotine was supplied very quickly acquired a violet-blue pigmentation which appeared to be localized in the vacuoles of the external cortical cells. No root injury of any sort was observed although the coloration was rather intense and in great contrast to the light cream color of the roots of the control plants. Some of the pigment was obtained in aqueous solution. It was insoluble in fat solvents, unchanged by zinc dust in dilute acid, but was destroyed by strong acid and strong base. The addition of silicotungstic acid to a portion of the original solution immediately led to destruction of the color and to the separation of a voluminous white precipitate. The supernatant solution was clear and colorless.

The second remarkable feature of these plants was the accumulation during the initial ten-day period of large amounts of nitrate nitrogen in the leaves and stems. Accompanying, or perhaps resulting from the accelerated nitrate uptake, was a proportionate increase in the reduced forms of nitrogen in root, stem and leaf (table I).

The plants that were supplied with tap water for a subsequent period of nineteen days gave further evidence of the changes that had been brought about. Under these conditions, the excess accumulation of nitrate largely disappeared, and a corresponding increase in other forms of water soluble and in hot-water-insoluble nitrogen fractions resulted. It was noted that the overall rate of nitrate assimilation during the period of nitrogen deprivation greatly exceeded that for ammonia assimilation (cf. **47**).

It was observed that the roots of the nicotine-fed plants accumulated relatively much ammonia and amide nitrogen, whereas the amount of nitrate stored in these organs did not appreciably change. Clearly, the overall response to the presence of extra nicotine, so far as nitrogen metabolism is concerned, was the absorption by the roots of abnormally large amounts of nitrate nitrogen from the nutrient solution.

If the view is adopted that nicotine may function within the tobacco root as an accelerator of nitrate absorption, it then becomes necessary to inquire

into the possible mechanisms through which such an activity might be exercised. From the physiological point of view, alkaloid synthesis could conceivably increase the concentration of hydroxyl ion derived from water and thereby make possible increased acid exchange between root and soil. Furthermore, the alkaloid could serve as a buffer substance in the roots against the accumulation of dangerous amounts of nitric acid or of its immediate reduction product, nitrous acid. These suggestions break down under the fact that the ratio of nicotine to nitrate absorbed was so exceedingly small. For instance, in the experiment under consideration, only 1.2 m.e. of nicotine (calculated as mono-acid base) were absorbed from the substrate and accumulated without change, while 28.7 m.e. of nitrate were

TABLE I

CHANGES IN THE NITROGENOUS FRACTIONS OF TOBACCO PLANTS AS A RESULT OF (1) THE ADDITION OF NICOTINE HYDROCHLORIDE TO THE NUTRIENT SOLUTION FOR TEN DAYS AND (2) SUBSEQUENT WITHDRAWAL OF NICOTINE AND OF MINERAL NUTRIENTS FOR NINETEEN DAYS. FIGURES ARE IN MILLIGRAMS OF NITROGEN PER PLANT

	Nitrogenous fractions					
	NH_3-N	Amide-N	NO_3-N	Nicotine-N‡	Unknown-N	Insoluble-N
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
(1) Nicotine supplied*						
Shoots	+20.2	+59.0	+119.4	+32.4	+ 35.8	+80.4
Roots	+13.1	+ 9.2	− 0.4	+ 1.0	+63.5	
(2) Nicotine withdrawn†						
Shoots	− 7.4	+ 4.0	−246.2	+ 2.8	+128.6	+83.0
Roots	−11.3	− 6.1	− 1.0	+ 0.6	−38.9	

* Figures express the differences between plants receiving nicotine for ten days and those receiving none.

† Figures express the differences between plants from which nicotine and all mineral nutrients were withdrawn for nineteen days and plants which had been supplied with both nicotine and mineral nutrients for an earlier ten-day period.

‡ During the period of nicotine feeding, each plant received 86.5 mgm. of nicotine nitrogen in 500 ml. of nutrient solution per day.

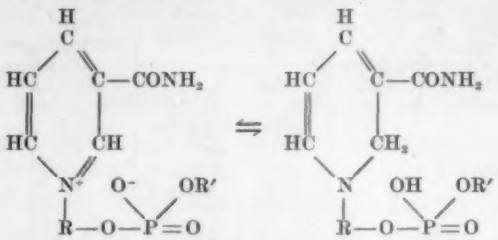
obtained from the same source (the solution contained no ammonium nitrogen).

A second possibility would seem to be that nicotine may alter the permeability of the root tip cells to the calcium, magnesium or potassium salt of nitric acid. This effect should be readily detected by ash analyses of the root, stem, and leaf tissues of the plants concerned.

The most suggestive possibility, however, would seem to follow the observation (table I) that, while much nitrate presumably traversed the root on its way to storage in leaf and stem, none was accumulated by the root cells as such. Instead, these cells contained relatively large amounts of ammonia, amide, and the undetermined forms of nitrogen including protein. While no estimate of the actual intensity of nitrate reduction in the roots is available from the existing data, it seems not unlikely that the presence of extra nicotine may have resulted in a greatly increased rate of reduction of nitrate to ammonia in the roots. This, in turn, may have conditioned an increase in

the rate of nitrate absorption by providing not only an extra supply of hydroxyl ion for acid exchange with the nutrient solution but also an equivalent amount of ammonium ion for preserving a favorable pH within the root cell fluids.

It seems worthwhile here to point out some of the chemical properties of nicotine by means of which effects such as those described above could be brought about. For instance, the nicotine molecule contains two feebly basic trivalent nitrogen atoms through which it is entirely conceivable that salt linkages might serve as points of attachment to specific proteins. The pronounced narcotic action of nicotine in the animal body lends weight to such a suggestion and, indeed, indicates the need for an investigation of the behavior of the alkaloid in both the animal and the plant cell from the point of view of the systems described by JOHNSON, EYRING, and WILLIAMS (**22**); JOHNSON, EYRING, and KEARNS (**21**); and MCELROY (**29**). If the narcotic action of nicotine in the animal body is based upon its ability to displace reversibly the prosthetic groups of one or more enzymes or upon its ability to denature reversibly the protein component of such enzymes, then the prospect must also be envisaged that nicotine or a metabolite of nicotine in

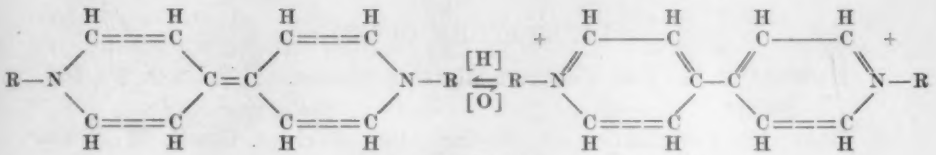


SCHEME II

combination with a specific protein may either catalyze or inhibit physiologically useful reactions in the tobacco root cells. In the event that evidence were forthcoming for such a mechanism, however, it would also be necessary to explain the fact that so much of the nicotine is lost from this combination and is permitted to be carried in the transpiration stream to those parts of the plant in which its physiological activity seems to be at a minimum [cf. the dissociation of nicotinamide-protein systems (**36**)].

The possible combination of nicotine (or of nicotine metabolites) with specific proteins raises many interesting possibilities for experimentation. For instance, although WARBURG has studied the properties of heme in combination with nicotine, no one seems to have attempted to obtain a biologically active substance by substituting nicotine for nicotinic acid amide in model substances related to the codehydrogenases. The codehydrogenases themselves are derivatives of pyridine, and their enormous biological importance needs no emphasis here (**36**). It is of interest to note, however, that the pyridine ring nitrogen of these compounds seems peculiarly fitted for the facile change from trivalency to pentavalency and back again as electrons are shifted from one catalyst to another in the course of cellular respiration (Scheme II). The general properties of pyridinium compounds and the

possibility of the existence of semiquinoid intermediates (**27**) in the reversible hydrogenation of nicotinic acid amide lead to the suggestion that, even in the absence of established combination with specific proteins, the potential biological importance of pyridine derivatives including nicotine deserves more widespread attention. From the point of view of the present discussion, the formation of quinoid structures such as those of the blue-colored N,N-dialkyldihydrodipyridyls is of great interest (**16**). The latter are easily oxidized with silver nitrate and less readily with atmospheric oxygen, followed by treatment with hydrochloric acid to form the dialkyl halogenide of 4,4′-dipyridyl. These, in turn, may be reduced back to the original blue mother compound with zinc dust and glacial acetic acid (Scheme III).



SCHEME III

While it seems likely that the pyridine ring in the nicotine molecule could contribute to such biochemical processes as the reduction of nitrate, it must not be overlooked that the pyrrolidine ring also possesses interesting properties. For instance, nicotine can be oxidized by mere exposure to air and to sunlight to the corresponding pyrrolidine-N-oxide containing a pentavalent nitrogen atom. With silver oxide in warm water, nicotine is dehydrogenated to nicotyrine (**2**). To carry speculations such as these to their logical conclusion, it becomes necessary to assume that electron shifts, in which nicotine or a metabolite of nicotine participates, could result either directly or indirectly in the absorption of relatively large amounts of nitrate ion from the soil solution without disturbing the electrostatic balance of the root tissue fluids. Whether or not this alkaloid, or any other alkaloid, can perform such a direct function in the plant that produces it remains to be determined by actual experimentation. These relationships are suggested here merely to call attention to the fact that the field is by no means devoid of problems for investigation.

Finally, it seems possible that careful study will reveal equally interesting but relatively minor biological rôles for nicotine in tobacco stem and leaf tissues. For instance, it is now definitely established that a certain small fraction of the nicotine content of excised leaves disappears as such during curing and during culture (**44, 46**). The extensive mobilization and disappearance of nicotine during fruiting and senescence has been noted above (**45**). Experiments have also been performed in which nicotine was supplied to excised tobacco shoots during culture in light (**8**). In all cases, approximately 25 per cent. of the nicotine absorbed by these shoots was converted into some other form of nitrogen which was water-soluble but not volatile with steam from alkaline solution. Certainly, the chemical nature

of the alkaloid would seem to fit it for many possible rôles in cellular metabolism including oxidations and reductions, both reversible and irreversible, and combinations with other compounds such as the proteins. It is, therefore, a great mistake to relegate it without further study to the scrap-heap of "waste-products" of tobacco metabolism and thence to consider the matter of little further importance.

The author is greatly indebted to Dr. W. G. Frankenburg, Director of Research for the General Cigar Company, for his kindness in critically reviewing the manuscript prior to its publication.

Princeton University
Princeton, New Jersey

LITERATURE CITED

1. Bernardini, L. Non-nicotine containing tobacco. Il Tabacca, No. **504**. 1938.
2. Blau, F. Constitution of nicotine. Ber. d. chem. Gesell. **27**: 2535–2539. 1894.
3. Broughton, J. Chemical and physiological experiments on living Cinchonae. Philos. Trans. Roy. Soc. London **161**: 1–15. 1871.
4. Cicerone, D., and Marocchi, G. Variation of nicotine content within a tobacco leaf. Bol. Tech. Coltiv. Tabacchi **12**: 119. 1913.
5. Cromwell, B. T. Experiments on the synthesis of hyoscyamine in *Atropa belladonna*. Biochem. Jour. **31**: 551–559. 1937.
6. Dawson, R. F. Nitrogen nutrition and nicotine synthesis in tobacco. Bot. Gaz. **100**: 336–346. 1938.
7. ———. Influence of certain amino acids and of nicotinic acid upon the nicotine content of tobacco leaves. Plant Physiol. **14**: 479–491. 1939.
8. ———. Metabolism of nicotine monohydrochloride in excised tobacco shoots. Amer. Jour. Bot. **27**: 190–194. 1940.
9. ———. The localization of the nicotine synthetic mechanism in the tobacco plant. Science n.s. **94**: 396–397. 1941.
10. ———. Accumulation of nicotine in reciprocal grafts of tomato and tobacco. Amer. Jour. Bot. **29**: 66–71. 1942.
11. ———. Nicotine synthesis in excised tobacco roots. Amer. Jour. Bot. **29**: 813–815. 1942.
12. ———. Accumulation of anabasine in reciprocal grafts of *Nicotiana glauca* and tomato. Amer. Jour. Bot. **31**: 351–355. 1944.
13. ———. On the biogenesis of nornicotine and anabasine. Jour. Amer. Chem. Soc. **67**: 503–504. 1945.
14. ———. An experimental analysis of alkaloid production in Nicotiana: the origin of nornicotine. Amer. Jour. Bot. **32**: 416–423. 1945.
15. Evtushenko, G. A. Interaction of stock and scion in Nicotiana. Yarovizatsiya **3**: 49–61. 1939.

16. FRANKLIN, E. C., and BERGSTROM, F. W. Heterocyclic nitrogen compounds. II A. Hexacyclic compounds: pyridine, quinoline, and isoquinoline. Chem. Rev. **35**: 77–277. 1944.
17. GORTER, A. Über die Nikotinbildung bei Nicotiana nach der Fütterung mit Prolin. K. Akad. Wetensch. Amsterdam, Proc. Sect. Sci. **39**: 87–90. 1936.
18. HASEGAWA, H. On some experiments in raising a nicotine-free tobacco plant. Botanic. Mag. (Tokyo) **51**: 306–316. 1937.
19. HIEKE, K. Pflanzenphysiologische Untersuchungen über die Alkaloide. II. Zur Alkaloidführung der Pfropfpartner bei der Heteroplastischen Solanaceenpfropfungen. Planta **33**: 185–205. 1942.
20. JACKSON, K. E. Alkaloids of tobacco. Chem. Rev. **29**: 123–197. 1941.
21. JOHNSON, F. H., EYRING, H., and KEARNS, W. A quantitative theory of synergism among diverse inhibitors, with special reference to sulfanilamide and urethane. Arch. Biochem. **3**: 1–31. 1943.
22. ————, ————, and WILLIAMS, R. W. The nature of enzyme inhibitions in bacterial luminescence: sulfanilamide, urethane, temperature and pressure. Jour. Cell. and Comp. Physiol. **20**: 247–268. 1942.
23. KLEIN, G., and LINSER, H. Zur Bildung der Betaine und der Alkaloide in der Pflanze. III. Vorversuche zur Bildung von Nikotin. Planta **20**: 470–475. 1933.
24. KRENKE, N. P. Wundkompensation, Transplantation, and Chimären bei Pflanzen. J. Springer. Berlin. 1933.
25. KUSMENKO, A. A., and TIKHVINSKAYA, V. D. Inheritance of Nicotine and anabasine content of *Nicotine tabacum* × *N. glauca* hybrids and interaction of stock and scion when these species are grafted. Bull. Acad. Sci. U.R.S.S. Biol. Ser. No. **4**: 564–567. 1940.
26. LIEBIG, J. VON. Die Chemie in ihrer Anwendung auf Agrikultur und Physiologie. Braunschweig. 1875.
27. LINDERSTROM-LANG, K. Enzymes involved in biological oxidation and reduction. Ann. Rev. Biochem. **6**: 44–49. 1937.
28. MARKWOOD, L. N. Nornicotine as the predominating alkaloid in certain tobaccos. Science n.s. **92**: 204–205. 1940.
29. MCELROY, W. D. The application of the theory of absolute reaction rates to the action of narcotics. Jour. Cell. and Comp. Physiol. **21**: 95–116. 1943.
30. MCMURTREY, JR., J. E., BACON, C. W., and READY, D. Growing tobacco as a source of nicotine. U. S. Dept. Agri. Tech. Bull. **820**. 1942.
31. MOTHES, K. Pflanzenphysiologische Untersuchungen über die Alkaloide. I. Das Nikotin im Stoffwechsel der Tabakpflanze. Planta **5**: 563–615. 1928.
32. ————, and HIEKE, K. Die Tabakwurzel als Bildungsstätte des Nikotins. Naturwiss. **31**: 17–18. 1943.
33. NATH, B. V. Scientific Reports of the Imperial Institute of Agricul-

tural Research, Pusa. 1934–1935. Report of the Imperial Agricultural Chemist. Delhi. 1936.

34. PAL, B. P., and NATH, B. V. Accumulation and movement of nicotine in reciprocal grafts between tobacco and tomato plants. Proc. Indian Acad. Sci. **20B**: 79–87. 1944.
35. PICTET, A. Über einige neue Pflanzenalkaloide. Ber. d. chem. Gesell. **40**: 3771–3783. 1907.
36. SCHLENK, F. Enzymatic reactions involving nicotinamide and its related compounds. Adv. Enzymol. **5**: 207–236. 1943.
37. SHMUCK, A. A. Changes in the chemical composition of plants upon grafting. Vsesouizaia akademiia sel'sko-khoziastvennykh nauk imeni V.I. Lenina, Moscow, Doklady **11**: 9–13. 1940.
38. ————, KOSTOFF, D., and BOROZDINA, A. Alteration in the alkaloid composition due to influence of stock upon scion in Nicotiana. Compt. rend. (Doklady) Acad. Sci. U.R.S.S. **25**: 477–480. 1939.
39. ————, SMIRNOV, A., and ILYIN, G. Formation of nicotine in plants grafted on tobacco. Compt. rend. (Doklady) Acad. Sci. U.R.S.S. **32**: 365–368. 1941.
40. SMITH, H. H., and SMITH, C. R. Alkaloids in certain species and interspecific hybrids of Nicotiana. Jour. Agri. Res. **65**: 347–359. 1942.
41. VICKERY, H. B. Chemical investigations of the tobacco plant. III. Tobacco seed. Part III. Some nitrogenous components of the hot water extract of fat-free tobacco seed meal. Connecticut Agri. Expt. Sta. Bull. **339**: 637–645. 1932.
42. ————. End products of nitrogen metabolism in plants. Biol. Symposia **5**: 3–19. 1941.
43. ————, and PUCHER, G. W. Chemical investigations of tobacco. Connecticut Agri. Expt. Sta. Bull. **311**: 234–246. 1930.
44. ————, ————, WAKEMAN, A. J., and LEAVENWORTH, C. S. Chemical investigations of the tobacco plant. Carnegie Inst. Wash. Publ. **445**. 1933.
45. ————, ————, LEAVENWORTH, C. S., and WAKEMAN, A. J. Chemical investigations of the tobacco plant. V. Chemical changes that occur during growth. Connecticut Agri. Expt. Sta. Bull. **374**: 557–619. 1935.
46. ————, ————, WAKEMAN, A. J., and LEAVENWORTH, C. S. Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. Connecticut Agri. Expt. Sta. Bull. **399**. 757–832. 1937.
47. ————, ————, ————, and ————. Chemical investigations of the tobacco plant. VIII. The effect upon the composition of the tobacco plant of the form in which nitrogen is supplied. Connecticut Agri. Expt. Sta. Bull. **442**: 65–119. 1940.
48. WINTERSTEIN, E., and TRIER, G. Die Alkaloide. 2d ed. Berlin. 1931.
49. WOLF, F. A., and GROSS, P. M. Flue-cured tobacco: a comparative study of structural responses induced by topping and suckering. Bull. Torrey Bot. Club **64**: 117–131. 1937.

NEW CARRIERS FOR PLANT GROWTH REGULATORS[1]

R. B. Withrow and F. S. Howlett

Lanolin has been used extensively as a carrier for indolebutyric acid for the production of parthenocarpic tomato fruits and for supplementing pollination and fertilization in the tomato. However, lanolin pastes are tedious to apply, they leave a sticky residue, and, in the early spring, the red or pink color does not develop evenly where such residues are excessive. Fruits produced following application of lanolin pastes and particularly lanolin emulsions are especially susceptible to the physiological disorder, blossom-end rot.

The writers have been concerned with possible substitutes for lanolin and for the emulsifying agent employed in the standard lanolin paste and lanolin-triethanolamine stearate emulsion (table IV). The ideal carrier for any growth-regulating substance should have the following characteristics: (1), low physiological activity in relation to the plant tissues involved; (2), physical stability so that the rate of release of the regulator is not greatly influenced by variations in environmental conditions such as temperature and moisture; (3), capacity to hold sufficient concentration of the regulator for optimum results, without the formation of large crystals or the occurrence of other forms of physical separation; (4), chemical inertness to the growth regulator so that the regulator is not chemically changed by the presence of the carrier; (5), ease of application to limited portions of the plant; and (6), low cost.

The procedure followed has involved the application of indolebutyric acid in various carriers to flowers of Globe Strain A tomato, both in the production of seedless fruits as well as in supplementing pollination and fertilization. In this report, only those carriers will be discussed which have given results comparable to those given by the standard lanolin emulsion. The bases of comparison used included (1) proportion of flowers setting fruit and the size attained by the fruits, (2) proportion of fruits from treated flowers developing blossom-end rot, (3) extent of the development of gelatinous pulp in the carpels, and (4) the ripening of such pulp. Data on the application of these carriers and fruit set occurring with them will be published in a subsequent paper.

Tissue injury tests with hydrocarbons, waxes, esters, and oils

In the search for substitutes for lanolin, tissue injury tests of a large number of hydrocarbons, waxes, esters, and oils were made on the leaves of tomato and soybean. Data on these tests, together with some of the properties of the various materials, are presented in table I.

[1] A joint contribution from the Purdue University and The Ohio Agricultural Experiment Stations. Journal Paper no. 204, Purdue University Agricultural Experiment Station.

Lanolin, at temperatures of 70° F. and below, caused no injury. At temperatures above 100° F., the leaves appeared oil-soaked and the tissues later died. Vaseline soaked into the leaf tissues a little more rapidly than lanolin and was somewhat more injurious at high temperatures. At low temperatures, it did not cause any injury. Paraffin oil killed the tissues and

TABLE I

PROPERTIES OF WAXES AND SIMILAR MATERIALS AS CARRIERS FOR INDOLEBUTYRIC ACID (IB) IN POLYVINYL ALCOHOL

WAX OR OIL	SOLVENT PROPERTIES FOR INDOLE-BUTYRIC ACID	POLYVINYL ALCOHOL EMULSION STABILITY	TISSUE INJURY	APPROXIMATE SOLIDIFYING POINT	REMARKS
				°C.	
Carnauba wax	Good	Very stable	None	85	Good carrier
Beeswax, white or yellow	Fair	Stable	None	62	Low activity of IB
Opal wax	Very good	Very stable	None	87	Low activity of IB
Wax blend no. 1	Very good	Stable	None	78	Good carrier
Wax blend no. 2	Very good	Stable	None	73	Good carrier
Spermaceti	Very good	Unstable	None	45	Good in mixtures with higher m.p. waxes
Lanolin, anhydrous	Good	Slightly unstable	Slight	30–40	Fair, except at high temperatures
Solid hydrocarbons	Poor	Stable	None	.....	Poor solvents for IB
Petrowax A				65	
Paraffin					
Ceresin					
Ozokerite					
Vaseline	Poor	Stable	Slight	.....	Poor solvent for IB
Paraffin oil	Poor	Stable	Severe	.....	Poor solvent for IB
Glyco waxes	Poor	Stable	.....		Poor solvents for IB
Glycerol esters	Good	Stable	Slight to severe	25	Injurious as a class
Cocoa butter				.....	
Bayberry wax				43	
Beef tallow					
Glycerol monostearate S, Glyco					
Cocoanut oil					
Olive oil					
Corn oil					
Cetyl alcohol	Very good	Unstable	None	49	Good in mixtures with other waxes
Stearic acid	Good	Unstable	Severe	69	

produced a water-soaked appearance. The solid hydrocarbons such as Petrowax A, paraffin, ceresin, and ozokerite resulted in no injury when mixed with sufficient lanolin for easy application to the leaves.

The true waxes such as carnauba, beeswax, and spermaceti caused no injury. Opal wax, a hard glycerol ester, was also non-injurious. The

glycerol esters such as cocoa butter, bayberry wax, beef tallow, and glycerol monostearate, induced varying degrees of injury when applied in mixtures with lanolin. Cocoanut, olive, and corn oils were very injurious to leaf tissues. Cetyl alcohol was quite inert and harmless.

Solubility for indolebutyric acid and other data for hydrocarbons, waxes, esters and oils

The solubility of indolebutyric acid was determined by adding the acid at the rate of 1.0 to 10 gm. of the material heated to about 100° C. If the crystals did not dissolve readily, the solubility was considered as low. Vaseline and the liquid and solid hydrocarbons are poor solvents for indolebutyric acid; the true waxes and glycerol esters are good solvents for indolebutyric acid. Beeswax and opal wax appeared to react with the indolebutyric acid so that the effectiveness of the acid was reduced in setting fruit.

Tissue injury tests with emulsifying agents

The data on tissue injury for a number of emulsifying agents are given in table II. The various grades of polyvinyl alcohol, the alkaline and acid

TABLE II

CHARACTERISTICS OF VARIOUS MATERIALS AS EMULSIFYING AGENTS FOR WAXES CONTAINING INDOLEBUTYRIC ACID (IB)

EMULSIFYING AGENT	EMULSIFYING PROPERTIES	TISSUE INJURY	TYPE OF EMULSION
Triethanolamine stearate	Excellent	Toxic*	Alkaline
Polyvinyl alcohol, high viscosity grade DuPont, PVA, RH–403	Very good	Nontoxic	Acid or alkaline
Polyvinyl alcohol, medium viscosity grade DuPont, PVA, RH–488	Good	Nontoxic	Acid or alkaline
Polyvinyl alcohol, low viscosity grade DuPont, PVA, RH–623	Very poor	Nontoxic	Acid or alkaline
Gelatin, pharmaceutical alkaline grade, Pharmagel B	Very poor	Nontoxic	Alkaline
Gelatin, pharmaceutical acid grade, Pharmagel A	Poor	Nontoxic	Acid
Methyl cellulose, low viscosity grade Dow Methocel, 15 cps	Poor for waxes; good for oils	Nontoxic	Acid or alkaline for oils only
Methyl cellulose, high viscosity grades Dow Methocel, 400 and 4000 cps	Poor	Nontoxic	
Bentonite clay	Poor	Nontoxic	Alkaline; leaves objectionable residue

* Injury tests determined with undiluted triethanolamine stearate. Toxicity not apparent when applied at low concentrations mixed with inert materials.

grades of pharmaceutical gelatin, high- and low-viscosity methyl cellulose, and Bentonite clay were all inert and produced no significant injury to leaf tissues when applied as aqueous mixtures. Triethanolamine stearate was very injurious to leaf tissue when applied undiluted. The components triethanolamine and stearic acid were also very injurious when applied separately.

Emulsification properties of agents

The low viscosity grades of methyl cellulose (**4**) form stable emulsions with oils but not with waxes. Methyl cellulose precipitates from solutions at the melting points of the waxes used. Bentonite clay does not form stable emulsions at concentrations sufficiently low to prevent objectionable residue on the fruit. Pharmagel A, derived from an acid-treated gelatin precursor, also formed unstable emulsions with the waxes used.

The high viscosity grades of polyvinyl alcohol and the pharmaceutical gelatin derived from an alkali-treated precursor, Pharmagel B, were the best

TABLE III

COMPOSITION OF CARNAUBA WAX AND LANOLIN MIXTURES

WAX NO.	COMPOSITION	
1	Carnauba wax	45%
	Lanolin, anhydrous	50%
	Cetyl alcohol	5%
2	Carnauba wax	25%
	Lanolin, anhydrous	70%
	Cetyl alcohol	5%

emulsifying agents for the waxes. Polyvinyl alcohol as an emulsifying agent is described by E. I. duPont de Nemours and Company (**5, 6**). The production of emulsions with pharmaceutical gelatins is described by TICE (**2, 3**) and the formulae given here involve the proportions of alkali recommended by him for Pharmagel B emulsions.

Emulsions

The blended waxes given in table III were the most suitable for the formation of emulsions. Carnauba wax used alone resulted in emulsions of relatively high viscosity. The two mixtures, wax blend no. 1 and wax blend no. 2, formed low-viscosity sprayable emulsions. The solidifying points of these blends are 78° and 73° C., respectively. Wax no. 2 is easier to keep liquefied in a Waring Blendor, but wax no. 1 appears to give slightly better results under high temperature conditions, and, therefore, it is recommended for use in cream emulsions for application at high temperatures.

Cetyl alcohol appeared to make the emulsions more stable when present in small amounts. Sorbitol was added to the emulsions as a nontoxic plasticizing agent to keep the polyvinyl alcohol or gelatin soft and capable of being stretched with the growth of the fruit. However, the authors have little

evidence thus far that the addition of such a plasticizer is of much value. Sodium bicarbonate, added to polyvinyl alcohol emulsions, keeps the emulsions alkaline, which appeared to increase the effectiveness of the indolebutyric acid, probably because it is more soluble in alkaline than in acid solutions. The pH of such emulsions is between 9 and 10.

Fluid emulsions

In table IV are presented the formulae for two typical stable fluid emulsions which have given satisfactory results in comparison with the standard triethanolamine stearate emulsion made with lanolin. Two different emulsifying agents are given. These emulsions can stand over considerable periods of time without breakdown. They are very fluid and easy to use without undue clogging of the atomizer used for their application.

TABLE IV

FORMULAE OF FLUID EMULSIONS

EMULSION TYPE	COMPOSITION
Standard lanolin triethanolamine stearate	0.2 gm. indolebutyric acid 1.3 gm. lanolin, hydrous 0.3 gm. triethanolamine 1.0 gm. stearic acid 100.0 ml. water
Wax blend no. 2–Pharmagel B	0.2 gm. indolebutyric acid 5.0 gm. wax blend no. 2 0.3 gm. Pharmagel B 0.1 gm. sorbitol 0.5 gm. sodium bicarbonate 100.0 ml. water
Wax blend no. 2–polyvinyl alcohol	0.2 gm. indolebutyric acid 5.0 gm. wax blend no. 2 0.5 gm. polyvinyl alcohol, RH–403 0.1 gm. sorbitol 0.5 gm. sodium bicarbonate 100.0 ml. water

PROCEDURE FOR THE PREPARATION OF FLUID EMULSIONS

The standard emulsion given for comparison in table IV may be prepared as described by HOWLETT (1). The other two liquid or sprayable emulsions were made according to the following procedure.

Two small openings were cut near the edge of the lid of a Waring Blendor jar. Through one opening was inserted an 8-mm. glass tube connected to a two-liter Erlenmeyer flask, half filled with water. The glass tube was so arranged that it was possible to slip it up and down vertically. Care was exercised to prevent the tube from catching in the blendor blades.

The wax was carefully melted in a beaker and brought to about 110° C. The indolebutyric acid was added next, keeping it just below 120° C., the point at which the acid crystals melt.

Forty per cent. of the water that was finally to be used was placed in the blendor jar; the dry polyvinyl alcohol, sorbitol, and sodium bicarbonate

were added and mixing begun. Heat was then applied to the Erlenmeyer flask and the blendor quickly brought up to 100° C. by violently boiling the water in the flask, injecting the steam into the blendor jar close to the mixing blades. A thermometer inserted in the second opening in the lid can be used for checking the temperature.

When the temperature reached 100° C., the melted wax–indolebutyric acid mixture was slowly added. Mixing was done with steam for about three minutes; the steam tube was then slipped out of the blendor jar; and the emulsion was mixed five minutes more. The most stable emulsions are obtained by allowing the blendor to finish mixing at slowest possible speed with the lid off until the mixture has cooled to about 60° C.

The contents of the blendor jar were poured into a flask which was shaken gently until all the bubbles on the surface were broken. The contents were then transferred to a graduate and made up to the indicated quantity with water.

Cream emulsions

Several cream emulsions having the consistency of lanolin were made up with other waxes mixed with lanolin. Wax no. 2 was found to make an excellent cream when Pharmagel B or polyvinyl alcohol was used as the emulsifying agent. Such creams do not leave sticky residues.

Procedure for preparation of cream emulsions

Formulae for the creams are given in table V. In preparing the cream emulsions, the indolebutyric acid was added to the melted wax as for fluid emulsions. All the water was heated to 100° C. and placed in a pre-heated Waring Blendor jar. The sodium bicarbonate and Pharmagel B or polyvinyl alcohol were then added. Next the blendor jar and its contents were

TABLE V

Formulae of cream emulsions

Paste type	Composition
Wax blend no. 1–Pharmagel B	0.2 gm. indolebutyric acid 20.0 gm. wax blend no. 1 2.0 gm. Pharmagel B 0.5 gm. sorbitol 0.5 gm. sodium bicarbonate 80.0 ml. water
Lanolin–Pharmagel B	0.2 gm. indolebutyric acid 20.0 gm. lanolin, anhydrous 2.0 gm. Pharmagel B 0.5 gm. sorbitol 0.5 gm. sodium bicarbonate 80.0 ml. water
Wax blend no. 1–polyvinyl alcohol	0.2 gm. indolebutyric acid 20.0 gm. wax blend no. 1 2.0 gm. polyvinyl alcohol, RH–403 0.5 gm. sorbitol 0.2 gm. sodium bicarbonate 80.0 ml. water

brought to 100° C. in a water bath. The melted wax mixture heated to 100° C. was then added. Mixing was done at high speed for 15 minutes with the jar thermally insulated, the temperature kept at 90° C. Care must be exercised so that the thermal insulation does not prevent the circulation of air past the motor of the blendor. Since the creams cannot be measured volumetrically with ease, a steam jet which adds an unknown amount of water cannot be used. The proper amount of water is added at the beginning, and no further additions should be made. The same procedure may be used for the fluid emulsions, but it takes more time than when a steam jet is used.

Mucilage solutions

Since indolebutyric acid converted to the sodium salt is soluble in water to the extent of several tenths of a per cent., it was thought that it might be

TABLE VI

CHARACTERISTICS OF VARIOUS MUCILAGES AND WATER-SOLUBLE SUBSTANCES FOR THE SODIUM SALT OF INDOLEBUTYRIC ACID

CARRIER	FILM FORMING PROPERTIES	REMARKS
Polyvinyl alcohol, high viscosity grade DuPont, PVA, RH–403	Tough, clear, flexible film	Good carrier, solutions clear, medium viscosity
Polyvinyl alcohol, medium viscosity grade DuPont, PVA, RH–488	Tough, clear, flexible film	Good carrier, solutions clear, medium viscosity
Polyvinyl alcohol, low viscosity grade DuPont, PVA, RH–623	Moderately tough, clear, flexible film	Very good carrier, solutions clear, low viscosity
Gum arabic	Weak, brittle film	Very good carrier, solutions clear, amber color, low viscosity
Carbowax, no. 1500 or no. 4000 (polyethylene glycol)	Soft film	Very toxic, good solvent for indolebutyric acid
Glycerine	Liquid	Very toxic, good solvent for indolebutyric acid, good plasticizer
Sorbitol	Non-film-forming	Nontoxic, poor carrier, good plasticizer

possible to eliminate the complicated preparation of emulsions by simply dissolving a concentrated alcohol solution of indolebutyric acid in an alkaline solution of a mucilage such as polyvinyl alcohol, gelatin, some of the natural gums as gum arabic, or of the polyethylene glycol sold as Carbowax by the Carbide and Carbon Chemicals Corporation. The properties of these materials are given in table VI.

Carbowax was soon eliminated, however, because it was very toxic to leaf tissues and tomato flowers. Leaves of tomato, sweet pepper, eggplant, melon, sweet potato, peach, apple, chrysanthemum, and rose were used for testing. When undiluted Carbowax was applied to leaf tissue, the tissues

were killed within four days on all the species tested. When mixed with 50 per cent. distilled water, the leaf tissues of all species were killed; 10 per cent. mixtures of Carbowax in distilled water injured the leaves of some of the species, especially sweet potato and eggplant. Mixtures containing 1 per cent. and less of Carbowax did not injure any of the leaf tissues. However, 1 per cent. mixtures severely injured tomato floral parts, and some burning occurred even at concentrations as low as 0.1 per cent. This was a disappointment because Carbowax had many interesting properties and was an excellent solvent for indolebutyric acid.

Gelatin formed solutions of high viscosity as in the case of the emulsions,

TABLE VII

FORMULAE OF MUCILAGE SOLUTIONS

SOLUTION TYPE	COMPOSITION
Glycerine–gum arabic	0.2 gm. indolebutyric acid 3.0 gm. glycerine 3.0 gm. gum arabic 0.5 gm. sodium bicarbonate 100.0 ml. water
Ethyl alcohol–gum arabic	0.2 gm. indolebutyric acid 3.0 ml. ethyl alcohol 3.0 gm. gum arabic 0.5 gm. sorbitol 0.5 gm. sodium bicarbonate 100.0 ml. water
Ethyl alcohol–polyvinyl alcohol	0.2 gm. indolebutyric acid 2.0 gm. ethyl alcohol 1.5 gm. polyvinyl alcohol, RH–403 0.1 gm. sorbitol 0.5 gm. sodium bicarbonate 100.0 ml. water

and it also tended to mold readily. Polyvinyl alcohol of the low viscosity RH–623 grade and gum arabic appeared to be the most suitable materials.

PROCEDURE FOR THE PREPARATION OF MUCILAGE SOLUTIONS

In table VII are given two formulae for mucilage solutions. In preparing these, the indolebutyric acid was dissolved in the alcohol. The sodium bicarbonate and sorbitol were dissolved in half the water in a Waring Blendor jar and the mucilage added. The solution was mixed at high speed until the mucilage was entirely dissolved. The solution was then heated with a steam jet to 100° C., the steam shut off, and the indolebutyric acid solution added. The remaining water was added, following cooling.

These solutions are clear and practically colorless. They are lower in viscosity than the emulsions and may be applied readily. Sufficient data are not as yet available to determine whether such solutions retain their activity during storage or whether they will prove as effective as the emulsions for use as carriers in setting fruit.

Discussion

Early in the work, it was thought that the increased incidence of blossom-end rot occurring with fruits treated with indolebutyric acid emulsions, as compared with untreated ones, was due to the toxicity of either the lanolin or triethanolamine stearate of the standard emulsion. However, as the work progressed, such increased blossom-end rot appeared to be only partly influenced by the carrier used. It appeared to be due primarily to the indolebutyric acid and to the rapid growth induced following its application. Under the environmental conditions which prevail in greenhouses in the northern United States at the time growth regulators are of value, lanolin and triethanolamine stearate usually are not visibly injurious to the floral parts at the dilutions used in the standard emulsion.

Summary

Formulae and methods of preparation of several stable fluid and cream emulsions employing waxes other than lanolin and emulsifying agents other than triethanolamine stearate are given. Mucilage solution formulae are also presented.

The cream emulsions do not leave sticky residues as does lanolin, and they are nontoxic to plant tissue. Tomato fruit set well when these emulsions were used as carriers for indolebutyric acid. Under conditions of high temperature where lanolin and lanolin emulsions are toxic, the materials recommended are not injurious. They also offer advantages where physiologically inert carriers are desired for materials other than growth regulating substances.

INDIANA AGRICULTURAL EXPERIMENT STATION,
LAFAYETTE, INDIANA,
OHIO AGRICULTURAL EXPERIMENT STATION,
WOOSTER, OHIO

LITERATURE CITED

1. HOWLETT, F. S. Growth-promoting chemicals improve greenhouse tomato production. Ohio Experiment Station, Bimonthly Bull. **28**: 17–27. 1943.
2. TICE, LINWOOD F. Gelatin as a stabilizing colloid for oil in water emulsion systems. Journal American Pharmaceutical Association **24**: 1062–1069. 1935.
3. ————————. The use of pharmaceutical gelatin. Publication of Edible Gelatin Manufacturers Research Society of America. 1945.
4. METHOCEL. Publication of the Dow Chemical Company, Midland, Michigan. 1940.
5. POLYVINYL ALCOHOL, PROPERTIES, USE AND APPLICATIONS. Publication of E. I. duPont de Nemours and Company, Wilmington, Delaware. 1940.
6. POLYVINYL ALCOHOL. PROCEDURE FOR EMULSIFYING "OPALWAX." Publication of E. I. duPont de Nemours and Company, Wilmington, Delaware. 1941.

THE ORGANIC ACIDS OF GRAPEFRUIT JUICE[1]

WALTON B. SINCLAIR AND D. M. ENY

(WITH ONE FIGURE)

The total acidity of grapefruit juice is highly associated with the quality of the fruit. Many of the biochemical reactions occurring in the fruit during growth and maturity depend upon the concentrations of the various acid constituents that compose the total acidity. The rates and types of reactions in citrus fruits are highly correlated with the pH, which in turn is directly related to the free and combined acids in the juice. These factors, considered collectively as the buffer system of the fruit, are important in regulating the proper reaction in the juice. The present investigation, therefore, is concerned with the determination of the organic acid constituents in grapefruit juice and the amounts of each that exist in the free and combined forms. Certain relationships are also noted between the pH and the existing cations that are available for salt formation.

Materials and methods

The methods of fruit sampling and the analytical procedures are the same as those used and described in similar studies on oranges and lemons (**7, 8, 9**). It is necessary to give only a brief listing of the procedures in this paper. Total soluble solids in the juice were determined as sucrose, with a Brix hydrometer. Using the table of STEVENS and BAIER (**10**), the true soluble solids were obtained by making a correction for the percentage of acid in the sample. The free-acid content of the samples was titrated on an aliquot portion of the juice with standard NaOH, with phenolphthalein as indicator. The organic acid fraction was precipitated from 80 per cent. alcohol with lead acetate and separated from the filtrate by centrifuging. The precipitate was washed with alcohol, suspended in water, and freed of lead by passing H_2S through the solution. The lead sulphide was filtered off and washed with water. The filtrate and washings were combined and diluted to a known volume. The citric and malic acids were determined simultaneously, on aliquot portions of this solution by the method of PUCHER, VICKERY, and WAKEMAN (**6**). The amount of organic acid in the combined form was estimated by determining the alkalinity of the ash from an aliquot of juice.

Results

ACID CONSTITUENTS OF GRAPEFRUIT JUICE PRECIPITATED WITH LEAD ACETATE

The organic polybasic acids of grapefruit juice were precipitated in 80 per cent. alcohol with lead acetate. An analysis of this lead-free precipitate gave the organic acids and acid salts naturally present in the juice. The

[1] Paper No. 535, University of California Citrus Experiment Station, Riverside, California.

TABLE I

ANALYSIS OF THE ORGANIC ACIDS OF GRAPEFRUIT JUICE

SAMPLE NO. AND LOCALITY	SAMPLING DATE	STAGE OF FRUIT MATURITY	FRUIT DIAMETER	TRUE* SOLUBLE SOLIDS	pH	FREE ACID (AS CITRIC) DETERMINED BY PHENOLPHTHALEIN TITRATION		ACID CONSTITUENTS PRECIPITATED BY LEAD ACETATE								COMBINED ACID (AS CITRIC) DETERMINED FROM ALKALINITY OF ASH		FREE ACID
								TOTAL ACID TITRATED (AS CITRIC)		CITRIC ACID (PENTABROMACETONE METHOD)		MALIC ACID		TOTAL ACID (CITRIC + MALIC)				
			cm.	%		*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	%
Fruit samples from packing house†																		
1. Riverside	July 17	Yellow	8.5	12.6	2.75	20.88	0.326	26.58	0.415	22.58	0.353	4.00	0.060	26.44	0.413	1.96	0.031	91.41
2. Riverside	July 19	"	8.5	12.5	2.80	21.51	0.336	26.58	0.415	23.58	0.368	3.30	0.049	26.70	0.417	2.08	0.032	91.18
3. Riverside	July 23	"	8.5	10.7	2.86	18.22	0.285	24.55	0.383	22.94	0.358	1.80	0.027	24.65	0.385	1.90	0.030	90.55
4. Sunnymead	July 26	"	10.0	10.3	2.80	18.48	0.289	24.90	0.389	23.48	0.367	1.62	0.024	25.03	0.391	2.02	0.032	90.14
5. Hemet	July 27	"	10.5	10.3	2.86	17.72	0.277	24.23	0.378	21.95	0.343	2.45	0.037	24.33	0.380	2.04	0.032	89.68
6. Riverside	July 31	"	10.5	11.0	2.86	17.53	0.274	23.44	0.366	21.32	0.333	2.30	0.034	23.50	0.367	1.96	0.031	89.94
7. Riverside	Aug. 3	"	8.0	10.8	2.87	17.34	0.271	23.28	0.364	20.27	0.317	3.46	0.052	23.62	0.369	1.71	0.027	91.02
8. West Riverside	Aug. 7	"	10.0	10.8	2.87	18.22	0.285	24.04	0.376	22.58	0.353	1.80	0.027	24.33	0.380	1.96	0.031	90.29
Fruit samples of different stages of maturity																		
9. Riverside	Aug. 20	Green	5.0	7.6	3.30	19.89	0.311	27.78	0.434	25.17	0.393	2.85	0.043	27.91	0.436	4.99	0.078	80.00
10. Riverside	Aug. 24	"	6.0	7.9	3.08	22.72	0.355	29.02	0.453	26.43	0.413	2.55	0.038	28.87	0.451	4.49	0.070	83.50
11. Riverside	Aug. 26	"	6.0	7.7	3.08	19.06	0.298	25.93	0.405	23.21	0.363	2.82	0.042	25.93	0.405	3.96	0.062	82.79
12. Indio	Aug. 28	"	7.5	10.5	2.96	22.84	0.357	29.03	0.453	25.90	0.405	2.97	0.044	28.74	0.449	3.03	0.047	88.29
13. Indio	Aug. 29	"	8.5	9.8	3.06	19.88	0.311	27.51	0.430	25.35	0.396	2.35	0.035	27.59	0.431	3.53	0.055	84.92
14. Indio	Aug. 31	"	9.3	9.9	3.08	22.84	0.357	30.29	0.473	26.08	0.407	4.10	0.061	29.96	0.468	3.28	0.051	87.44

* Due to the relatively high acid content of the juice, a correction was made on the hydrometer readings to give the true percentage of soluble solids (**10**).

† The high acid concentration and small sizes of the fruit in these samples, caused by the late season, indicate that the fruit is immature.

completion with which lead acetate precipitated the organic acids is made evident by the close agreement of the total acid values with those reported for the sum of the citric and malic acids (table I). The total acid values (as citric) represent all of the acid groups precipitated from the juice with lead acetate. That inorganic acid salts are not present in the juice in appreciable amounts is shown by the fact that the citric and malic acids account for all of the acid groups in the precipitate. These results do not agree with those of MENCHIKOVSKY and POPPER (**5**), who reported that the grapefruit of Palestine contained, in addition to citric and malic acids, small amounts of tartaric and oxalic acids.

The citric and malic acid contents of the juice from grapefruits of different maturities are shown in table I. It should be pointed out that these values, as determined by the method of PUCHER, VICKERY, and WAKEMAN (**6**), include both the free and combined acids in the form of citrates and malates. As in the lemon and the orange, the actual amount of citric acid in the juice represents the major portion of the total organic acid content of grapefruit. The decrease in concentration of the free acids with the fruit maturity is due chiefly to the citric acid content. In the samples studied, the concentration of malic acid and malate showed a variation of 100 per cent. No explanation can be given for such large variations in grapefruit juice. Large differences of this sort were not encountered in mature oranges and lemons. No evidence was obtained from these investigations which would indicate the existence of an equilibrium between the concentrations of citric and malic acids.

RELATION OF pH TO THE FREE AND COMBINED ACIDS IN GRAPEFRUIT JUICE

The concentration of free acids of mature grapefruit juice is between that of the lemon and the orange; and, consequently, the pH is similarly related. As in oranges, the amount of free acids (mg. per fruit) increased and reached a maximum in grapefruit during the early growth period, but the decrease in concentration of free acids (mg. per ml.) during ripening was caused evidently by the growth of the fruit and the consequent dilution of the acids present; for as the grapefruit increases in size the acids must be distributed through an increasing volume of juice.

The relation of pH to the concentration of free acid in different samples of grapefruit juice is shown in table I. In the immature fruit, the amount of combined acids varies from 12 to 20 per cent. of the total acid radicles, and while the acidity is near its maximum, the pH at this point is strongly influenced by the high salt content. In the mature fruit, the free acid varied from 17.34 to 21.51 mg. per ml.; the percentage of the total acid in the free state showed little variation. This means that the combined acids (salts of inorganic cations) did not vary enough to produce more than a slight shift in pH. The average amount of organic acid in the combined form was 1.95 mg. citric acid per ml., and this amount corresponds, on the average, to a salt content of 10 per cent. of the total acid radicles.

The pH-salt relationships of grapefruit juice are illustrated in figure 1 by comparing the titration curve of the juice with that of a pure citric acid solution. The solution used in securing the data for the citric acid curve contained 17.94 mg. of citric acid per ml.; the free acid in the grapefruit juice was 16.53 mg. per ml. The pure citric acid solution had a pH of 2.25; the grapefruit juice had an initial pH of 2.87. The initial point on the grapefruit juice curve was located on the graph at pH 2.87, which corresponded to a combined acid or salt content of 10 per cent. The percentage of acid neutralized for all points, other than the initial one, included the combined acid present in the original sample and the sodium citrate formed by the neutralization of part of the free acid. The close similarity of these two curves is indicated by the pH values of grapefruit juice, which are slightly

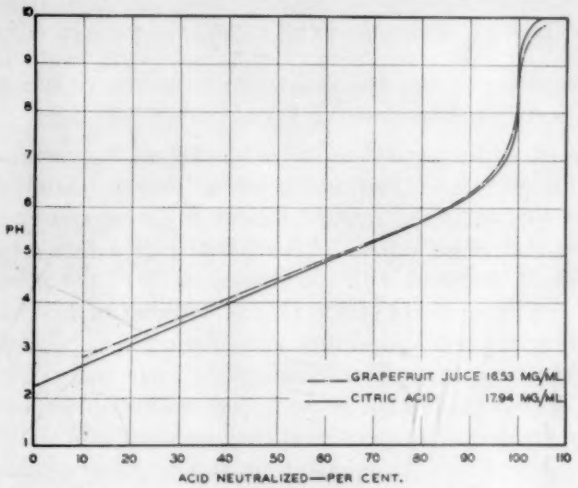


FIG. 1. Titration curves of grapefruit juice and of pure citric acid solution, showing the relation of pH to the salt content. The initial point on the grapefruit-juice curve was corrected for the salt occurring normally in the juice.

higher than those of citric acid, for a given amount of combined acid (salt). The two curves are nearly parallel and consequently have nearly the same slope. Grapefruit juice titrates like a pure citric acid solution because the free acid in the juice is composed chiefly of citric acid, and the ratio of $\frac{\text{free acid}}{\text{combined acid}}$ is sufficiently great to diminish the salt effect on the pH. The quantity of cations that are available in the juice for combining with the organic acids is relatively small in comparison with the concentration of free acids. To plot an accurate titration curve of grapefruit juice, a correction must be made for the combined acid naturally occurring in the juice. Grapefruit juice, like all weak acids, is most effective at a pH approximating the dissociation constant of the buffer acid (pKa).

During the ripening of grapefruit, the concentration of acid in the free state decreased, and the pH of the juice increased. The rise in pH was the

result of the change in the value log $\frac{\text{salt}}{\text{acid}}$, caused chiefly by the decrease in acid concentration. The combined acid reached a near constant value in the mature fruit. The difference in pH of two given samples of grapefruit juice usually amounted to the difference in the log $\frac{\text{salt}}{\text{acid}}$ values of the two samples. Similar results were obtained with the calculations made from the data of SINCLAIR and RAMSEY (9), which showed that juices from a green and mature orange-fruit sample, picked 7 months apart, had a difference in pH of 0.3. This change in pH was almost entirely accounted for by the corresponding difference (0.2838) in the log $\frac{\text{salt}}{\text{acid}}$ values of the two samples. These experimental results are theoretically derived from the equation which relates the pH to the dissociation exponent (pKa) and the ratio of salt to free acid.

THE ALKALINITY OF THE ASH AS A MEASURE OF THE COMBINED ACIDS AND THE BASE-FORMING ELEMENTS OF THE JUICE

The combined acids recorded in tables I to III were determined from the alkalinity of the juice ash. During the ashing process, the organic radicles are burned off leaving the equivalent cations as carbonates and oxides and some sulphates and phosphates. This alkalinity is a fair measure of the amount of cations combined with the organic acids in the juice. The concentration of combined acids (table II), as determined by this method, is greater in immature than in mature grapefruit juice. Juices of mature lemons and grapefruit have approximately the same concentration of combined acids. In mature orange juice, the concentration of combined acids is significantly greater. The specific cations combined with the organic acids have not been determined. It is highly probable, however, that the citrates (salts) are present in the juice as the potassium acid citrates, since the potassium concentration accounts for 60 to 70 per cent. of the total cations.

TABLE II

THE COMBINED ORGANIC ACIDS IN CITRUS JUICES

STAGE OF MATURITY	COMBINED ORGANIC ACIDS (AS CITRIC) IN*					
	GRAPEFRUIT		ORANGES		LEMONS	
	mg./ml.	*m.e./ml.*	*mg./ml.*	*m.e./ml.*	*mg./ml.*	*m.e./ml.*
Immature	4.49	0.070	2.75	0.043	1.83	0.028
"	3.96	0.062	2.74	0.043	1.90	0.029
"	3.03	0.047	2.68	0.042	1.79	0.028
"	3.53	0.055	2.50	0.039	1.88	0.029
"	3.28	0.051	2.75	0.043	1.86	0.029
Mature	1.96	0.031	2.92	0.046	2.13	0.033
"	2.08	0.032	3.01	0.047	2.08	0.032
"	2.02	0.031	3.07	0.048	2.13	0.033
"	2.04	0.032	2.94	0.046	1.94	0.030
"	1.96	0.031	2.88	0.043	2.15	0.033

* Determined from the alkalinity of the ash.

Extensive nutritional research has shown that citrus fruits (and others, such as tomatoes, pears, peaches, apricots, etc.) contain base-forming elements, which, when eaten and digested in the human body, have the power to neutralize the acids and, consequently, raise the pH of the urine. Although these fruits are acid in reaction, the degree of acidity or basicity in the metabolic process depends upon the extent to which the organic acid radicles are oxidized in the body, setting free cations (mostly potassium) to combine with the acid residues in the urine. When the body has an excess of basic elements to be eliminated, the ammonia secretion is greatly reduced and the organic acids combine with an equivalent of fixed base. The whole is excreted as salts which at the pH of the blood are neutral and consequently exhibit slight buffer capacity. Most fruit acids, and especially citric acid, are nearly completely oxidized in the body. Citrus juices, therefore, would not exhibit an ultimate acid effect, but they would reduce the

TABLE III

THE CATIONS COMBINED WITH THE ORGANIC ACIDS IN THE JUICE OF MATURE GRAPEFRUITS AND ORANGES

FRUIT VARIETY	TOTAL CATION CONTENTS*	CATION COMBINED WITH ORGANIC ACIDS†		CATION COMBINED WITH INORGANIC ANION
	m.e. 100 gm.	*m.e. 100 gm.*	%	*m.e. 100 gm.*
Grapefruit	5.35	3.05	57.00	2.30
Valencia oranges	5.96	4.26	71.47	1.70
Navel oranges	6.06	4.42	72.94	1.64

* The total cation content is the sum of the Ca, Mg, K, and Na, in the juice.
† Percentage of total cation.

acidity of the urine because of the alkaline ash produced during metabolism. This factor has been definitely established by the investigations of BLATHERWICK and LONG (**1**). The rapid rate at which citric acid is metabolized was demonstrated by KUYPER (**4**) in experiments which showed that the alkali formed from digested sodium citrate was excreted in the urine at approximately the same rate as that of sodium bicarbonate. From these considerations, the deduction can be drawn that the alkalinity of the ash is a measure of the base-forming capacity of the juice, as well as a measure of the cations combined with the organic acids.

It can be seen from table II that the base-forming capacity of mature grapefruit and lemon juices is equivalent to approximately 0.032 milliequivalents per ml.; for orange juice, the base-forming capacity is greater and amounts to approximately 0.046 milliequivalents per ml. For 100 grams of juice, the base-forming capacity of Valencias, navels, and grapefruit amounted to 4.26, 4.42, and 3.05 milliequivalents, respectively (table III).

The fraction of the total cations in the combined form in mature grapefruit juice is compared, in table III, with similar values for Valencia and navel oranges. The total cation content of the different juices is represented

by the sum of the calcium, magnesium, potassium, and sodium values expressed in milliequivalents per 100 grams of juice. Undoubtedly, other cations are present in the juice in micro-quantities which would produce only a slight effect on these values. Grapefruit juices had 57.00 per cent. of their total cations combined with the organic acids, and Valencia and navel orange juices had 71.47 and 72.94 per cent., respectively. The remainder of the cations are combined in the juices with the inorganic anions, which include sulphates, chlorides, nitrates, and part of the phosphates. The titration curve of phosphoric acid demonstrates that approximately 34 per cent. is in the salt form (KH_2PO_4) at pH 4.60. Since mature grapefruit and orange juices have a pH 3.00 to 3.70, approximately 25 per cent. of the total phosphorus is in the salt form. Lemon juice, which has a pH of 2.1, has from 12 to 15 per cent. of the phosphorus existing as a salt.

It is apparent that the amount of organic acids that combine with the mineral bases to form salts depends upon the concentration of available cations. The amount of free organic acids available for salt formation in the juice would never be a limiting factor, for the acid concentration at all times would be many times greater than the total cation content. Although the total cation content of a given sample is derived from the ash, the concentration in the juice is correlated with the mineral elements absorbed by the roots during fruit development. Soil conditions favorable for increased absorption of minerals by the plant could produce an increase in the total ash of the fruit, thereby increasing the alkalinity of the ash in the juice. The total ash content of the fruit can apparently be increased only to a limited extent by differences in cultural conditions. The total mineral constituents in vegetative portions of most plants usually respond to climatic and soil changes more readily than do those in the fruit. It can be seen from the titration curve of grapefruit juice (fig. 1) that a 100 per cent. increase in the total cation content in the juice would increase the combined acid only 10 per cent. This increase in combined acids is based, of course, on the supposition that the alkalinity of the ash increased in proportion to that of normal juice. A 10 per cent. decrease in free acid of mature grapefruit juice would cause an increase of only 0.3 of a pH (table I).

It is fully realized that the rate of formation of the free acid in grapefruit and oranges is greatly reduced by various sprays such as arsenates (**3**) and phosphates (**2**). Those sprays are most effective when applied to the trees at the early stage of acid accumulation before the free-acid concentration has reached a maximum in the fruit. Since the decrease in concentration during the maturation period is relative to the increase in volume, it would be interesting to study the effect of sprays applied at this period.

Summary

The organic acids of grapefruit juice are citric and malic acids. The total acid content precipitated from grapefruit juice by lead acetate is equal to the sum of the citric and malic acids. The titration curve of grapefruit

juice is very similar to that of a pure citric acid solution, provided a correction is made for the combined acid naturally occurring in the juice. There is a definite relation between the pH and the amount of acid in the salt or combined form. The small fluctuations in pH of mature grapefruit juice are correlated with the large ratio of free acid/combined acid.

The free acids decreased and the pH of the juice increased with increase in fruit size. The decrease of the free-acid concentration in grapefruit during development is due chiefly to the increase in fruit size rather than to a change in the absolute amount of free acid per fruit.

The alkalinity of the ash is a measure of the base-forming capacity of the juice, as well as a measure of the cations combined with the organic acids. In grapefruit juice, more than 50 per cent. of the total cations are utilized in the formation of the organic acid salts, as compared to more than 70 per cent. in the juice of Valencia and navel oranges. A large increase in available cations from soil fertilization would produce a limited increase in combined acid and little decrease in juice acidity.

UNIVERSITY OF CALIFORNIA
CITRUS EXPERIMENT STATION
RIVERSIDE, CALIFORNIA

LITERATURE CITED

1. BLATHERWICH, N. R., and LONG, M. L. Studies in urinary acidity. I. Some effects of drinking large amounts of orange juice and sour milk. Jour. Biol. Chem. **53**: 103–109. 1922.
2. ESSELEN, D. J., and OBERHOLZER, P. C. J. Reduction of acid in Valencias. Farming in So. Africa **14**: 21–22. 1939.
3. HARDING, PAUL L., and FISHER, D. F. Seasonal changes in Florida grapefruit. U. S. Dept. Agr. Tech. Bul. **886**. 1945.
4. KUYPER, A. C. The rate of excretion of ingested acid and alkali. Jour. Biol. Chem. **145**: 615–619. 1942.
5. MENCHIKOVSKY, F., and POPPER, S. Organic acids in Palestinian grapefruits. Hadar **5**: 181–183. 1932.
6. PUCHER, G. W., VICKERY, H. B., and WAKEMAN, A. J. Determination of malic acid in plant tissue. Simultaneous determination of citric and malic acids. Ind. & Eng. Chem., Anal. Ed. **6**: 288–291. 1934.
7. SINCLAIR, W. B., BARTHOLOMEW, E. T., and RAMSEY, R. C. Analysis of the organic acids of orange juice. Plant Physiol. **20**: 3–18. 1945.
8. ————, and ENY, D. M. The organic acids of lemon fruits. Bot. Gaz. (In press.) 1946.
9. ————, and RAMSEY, R. C. Changes in the organic-acid content of Valencia oranges during development. Bot. Gaz. **106**: 140–148. 1944.
10. STEVENS, J. W., and BAIER, W. E. Refractometric determination of soluble solids in citrus juices. Ind. & Eng. Chem., Anal. Ed. **11**: 447–449. 1939.

EFFECTS OF VARIOUS AGENTS ON THE STRUCTURAL VISCOSITY OF ELODEA PROTOPLASM[1]

HENRY T. NORTHEN

Ethylene chlorhydrin, thiourea, and the fat solvent anesthetics, when used in certain concentrations and exposures are often effective in breaking rest periods in plants (**8, 13**). These agents may also modify the rates of respiration and polysaccharide hydrolysis. The rates of such processes are frequently increased when plants are treated with relatively low concentrations and decreased when treated with higher ones (**3, 6, 7, 8, 10, 13, 14, 17, 18, 19**). It has been suggested (**23**) that the breaking of rest periods and accelerated rates of respiration, polysaccharide hydrolysis, imbibition, and permeability are a consequence of a dissociation (disaggregation) of protoplasmic proteins and that the retarded rates which result from longer exposures or higher concentrations are conditioned by an association (aggregation) of protoplasmic proteins.

The data presented later indicate that in certain concentrations ethylene chlorhydrin, thiourea, and the fat solvent anesthetics diminish the structural viscosity of protoplasm in leaf cells of Elodea. Decreases in structural viscosity are presumed to result from a dissociation of protoplasmic proteins (**23**).

Exposures to low temperatures may also influence plants by breaking rest periods (**4**) and altering the course and rate of development (vernalization). It will be shown that a temperature of 3° C. conditions a decrease in the structural viscosity of Elodea protoplasm.

As yet there is no general agreement as to the rôle of copper and zinc in the metabolism of plants, although it is known that in traces they accelerate one or more plant activities and in larger quantities they are toxic (**2**). In relatively low concentrations these metals lower protoplasmic viscosity whereas in higher concentrations they increase it.

Materials and methods

For each experiment (tables I and II) one whorl of Elodea leaves was placed in tap water, which was used except as specified in the tables, and the adjacent whorl was placed in the experimental solution made up with tap water unless otherwise indicated. The above procedure was repeated with three other plants, thus giving a sample of four whorls of leaves for the experimental group and an equal number for the control group.

After the desired time of immersion at room temperature (approximately 21° C.), the control and experimental whorls were placed on cotton wet with water for the controls and the appropriate solution for the experimental group. After inserting the cotton wads in centrifuge tubes, the leaves were

[1] Contributions from the Department of Botany and the Rocky Mountain Herbarium of the University of Wyoming, no. 200.

centrifuged with an acceleration of 680 × gravity for 90 seconds, an acceleration which usually displaced the chloroplasts only in cells at the base of the control leaves. In each experiment the control and experimental groups were centrifuged synchronously. Following centrifugation one leaf from

TABLE I

EFFECTS OF VARIOUS AGENTS ON THE STRUCTURAL VISCOSITY OF ELODEA PROTOPLASM

AGENT	CONCENTRATION	PERCENTAGE DIFFERENCES BETWEEN CONTROL AND EXPERIMENTAL GROUPS WHEN EXPOSED FOR			
		15 MIN.	30 MIN.	60 MIN.	120 MIN.
Ethylene chlorhydrin (anhydrous)	0.5%	.....	−36*	−36	−35
	1.0	−53	−68	−35	.....
	2.0	−72	−60	−91	−69
	5.0	+15	+12	+15	+18
	10.0	+12	+15	+12	+15
Thiourea	1.0%	−32	− 3	+ 3	0
	2.0	−67	−62	−63	−42
	3.0	−79	−95	−87	.....
Ether (anhydrous)	2.0%	−38	−40	−24	−40
	3.0	−56	−42	−45	−52
	4.0	−80	−71	−53	.....
	6.0	+ 2	+13	+ 6	0
Ethyl alcohol	3.0%	−57	−42	−50	−52
	6.0	−55	−86	−72	−64
	12.0	−46	−14	− 3	−24
	15.0	+39	+40	+42	.....
Propyl alcohol (normal)	1.75%	−85	−51	−64	−20
	3.50	−82	−94	−88	−83
	7.00	+ 8	+14	.....	+15
Butyl alcohol (normal)	0.75%	−67	−57	−35	−21
	1.50	−83	−84	−74	−54
	3.00	+14	.....	.....	.....
Sucrose	0.6 M	−95	−99	−97	−92
KCl	0.3 M	−84	−95	−97	.....
$CuCl_2 \cdot 2H_2O$ in distilled water	0.00001 M	− 3	− 3	+ 2	− 5
	0.00005	− 1	−12	−26	−38
	0.0001	− 8	−41	−62	−47
	0.001	−70	−60	+ 6	+14
	0.01	+ 9	+ 8	.....	.....
$CuCl_2 \cdot 2H_2O$ in tap water	0.0001 M	−72	−65	−60	−26
	0.001	−78	−53	.....	+ 5
	0.01	− 1	0	.....	.....
$ZnSO_4 \cdot 7H_2O$ in distilled water	0.0001	− 2	− 3	0	−38
	0.0005	−10	−16	−28	−26
	0.001	− 1	− 7	−28	−47
	0.01	−17	−41	−35	+ 1
Temp. of 3° C.	.....	−84	−77	−85	−81

* Minus (−) indicates a decrease in viscosity; plus (+) an increase.

each of the four whorls of the experimental and control groups was mounted in seventy per cent. alcohol. For the first trials two leaves from each whorl were mounted, but as the two leaves gave similar results in later experiments only one leaf from each whorl was used. Subsequently the approximate

percentages of cells in which the chloroplasts had been displaced (moved to the centrifugal wall) were determined. Each experiment was repeated at least once and the leaves, following centrifugation, were examined to determine if there was general agreement with the previous trial. The repetitions gave comparable results in all instances.

Results

The data are summarized in tables I and II. Each figure in the columns represents the difference between the average percentage of displacement noted in the control group and in the corresponding experimental group. For example, the average percentage of cells in which the chloroplasts were displaced in the four water control leaves which were centrifuged at the same time as the four leaves which had been treated with 0.5 per cent. ethylene

TABLE II

EFFECTS OF VARIOUS AGENTS ON THE STRUCTURAL VISCOSITY OF ELODEA PROTOPLASM

AGENT	CONCENTRATION	PERCENTAGE DIFFERENCES BETWEEN CONTROL AND EXPERIMENTAL GROUPS WHEN EXPOSED FOR			
		1 MIN.	2 MIN.	5 MIN.	10 MIN.
Ethylene chlorhydrin	5.0%	−67	−68	−64	+25
Ether	6.0%	.....	.....	−30	.....
Ethyl alcohol	15.0%	.....	−78	−65	−42
	18.0	−54	−45	+13	+25
Propyl alcohol	7.0%	.....	−89	−78	−82
Butyl alcohol	3.0%	.....	−93	−30	.....
$CuCl_2 \cdot 2H_2O$ in distilled water	0.01 M	.....	−26	−17	.....

chlorhydrin for 30 minutes was 12 per cent. whereas in the leaves treated with ethylene chlorhydrin the chloroplasts were displaced in 48 per cent. of the cells. The difference, 36 per cent., is the figure recorded in the table. Negative figures indicate that there was less displacement in the controls than in the experimental leaves and positive numbers indicate that the displacement was greater in the controls. Accordingly negative values indicate decreases in protoplasmic viscosity and positive ones increases.

The data in tables I and II disclose that ethylene chlorhydrin, thiourea, ether, ethyl alcohol, propyl alcohol, butyl alcohol, cupric chloride, and zinc sulphate lower the structural viscosity of the protoplasm when used in relatively low concentrations or relatively high concentrations for short periods of time. On the other hand, when higher concentrations were used, except with thiourea, the initial decrease was followed by an increase to at least normal and in some cases to higher than normal.

The 0.6 molal sucrose and 0.3 molal potassium chloride plasmolyzed the cells. Such plasmolysis induced a striking decrease in the viscosity of the protoplasm.

The viscosity was much less in cells maintained at 3° C. for periods ranging from 15 minutes to 2 hours than in cells maintained at room temperature, 21° C. Cells maintained and centrifuged at 3° C. invariably showed a greater percentage of chloroplast displacement than did the controls (those maintained at 21° C.), which were centrifuged at the same time.

Discussion

Long ago HUXLEY suggested that protoplasm, wherever located, was similar in many particulars. If this be true, the living substance, irrespective of the body in which contained, may respond in like manner to applied agents.

Fat solvent anesthetics affect protoplasm in various organisms in a similar manner, decreasing the protoplasmic viscosity in certain concentrations and increasing it in higher ones (**5, 11, 12, 26**). Some of the concentrations and exposures used in this investigation were the same as those used by DAUGHERTY (**5**) in her studies of Amoeba protoplasm. The results, decreased viscosities, obtained by her for the cortical gel of Amoeba are like those obtained for Elodea when concentrations of 6 per cent. ethyl alcohol, 3.5 per cent. propyl alcohol, 1.5 per cent. butyl alcohol, and 2 per cent. ether were used. Daugherty did not use higher concentrations. When Elodea cells were treated with high concentrations an initial decrease was soon followed by an increase in viscosity, and such results are like those obtained with Spirogyra (**20**).

With respect to the effects of copper on protoplasmic viscosity, the only previous complete study, one in which several concentrations were used for different intervals, is that of ANGERER (**1**) who noted that with concentrations of cupric chloride less than 10^{-3} molar the protoplasmic viscosity of sea urchin eggs decreased after a latent period whereas with longer time intervals the viscosity increased. Because the protoplasm of Elodea reacted likewise, copper probably affected these apparently unrelated protoplasms in a similar manner.

TIMMEL (**25**) working with Caltha and NORTHEN (**22**) with Spirogyra previously noted that plasmolysis decreases protoplasmic viscosity, a result in agreement with that presented in this paper.

In contrast to the similar responses of different kinds of protoplasm to fat solvent anesthetics, cupric chloride, and hypertonic solutions, the response of protoplasm to low temperature is not identical in the various organisms that have been studied or the methods of applying low temperatures have not been the same. According to HEILBRUNN (**12**) some types of protoplasm show a regular increase in viscosity with diminishing temperature whereas other types may exhibit a decrease in viscosity as the temperature is lowered in a given range. The protoplasm of Spirogyra is probably of the first type (**21**) whereas the cortex of *Amoeba proteus* is an example of the second type. When Amoeba were maintained in culture fluid the viscosity increased as the temperature was raised from 3° C. to 7° C., but with

further increase up to 33° C. the viscosity decreased (**24**). However, at 20° C. the viscosity was higher than at 3° C., a result which is in agreement with that obtained for Elodea.

Ethylene chlorhydrin and thiourea in appropriate concentrations decrease the structural viscosity of Elodea protoplasm (tables I and II). The data of MARCY (**16**), who studied the effects of ethylene chlorhydrin and thiourea on protoplasmic streaming, may be considered in accord with the data presented. She noted an acceleration of protoplasmic streaming in cells of Nitella and Elodea which had been immersed for 2 hours in 1 per cent. ethylene chlorhydrin and 1 per cent. thiourea. Such increased streaming may indicate that the viscosity of the protoplasm had been decreased.

Many of the chemicals used in this investigation not only affect protoplasm, but they also influence the rates of such processes as respiration, polysaccharide hydrolysis, imbibition, and permeability, generally conditioning increased rates when used in appropriate concentrations and decreased rates when used in higher ones (**3, 6, 7, 8, 9, 10, 13, 14, 15, 17, 18, 19**). In addition many of the agents are effective in breaking rest periods. It is believed (**23**) that the agents do not directly affect the processes mentioned but that the rates are influenced by the alterations of protoplasm, being increased when the protoplasmic viscosity is lowered and decreased when the viscosity is increased.

The decreases in structural viscosity recorded in this paper probably result from a dissociation of the protoplasmic proteins (that is, the splitting of large protein molecules into units of smaller size). NORTHEN *hypothesized* (**23**) that such dissociation often conditions increases in the rates of such processes as respiration, imbibition, permeability, polysaccharide hydrolysis, and under some conditions the dissociation may be primarily responsible for the breaking of rest periods. The idea that a disaggregation of cell colloids (resulting in a greater dispersion) hastens the rates of many cellular processes is a rather old and well-established hypothesis. NORTHEN's concept augments this older idea in that he recognizes that dissociation not only increases the surface, but it may also result in the liberation of enzymes from their previous combinations and in the formation of enzyme activators such as sulfhydryl groups.

Summary

The centrifuge method was used to determine the effects of various agents on protoplasmic viscosity in cells of Elodea. Concentrations of 2 per cent. ethylene chlorhydrin, 3 per cent. thiourea, 4 per cent. ether, 6 per cent. ethyl alcohol, 3.5 per cent. propyl alcohol, 1.5 per cent. butyl alcohol, 0.0001 molal cupric chloride, and 0.001 molal zinc sulphate conditioned decreases in the structural viscosity when the exposures were two hours or less. Decreases in viscosity were presumed to result from dissociations of protoplasmic proteins. Concentrations one-half of those listed above also decreased the viscosity, while with concentrations greater than those mentioned an initial decrease in viscosity was usually followed by an increase.

Plasmolysis with 0.6 molal sucrose or 0.3 molal potassium chloride induced a decrease in viscosity.

The protoplasmic viscosity was lower at a temperature of 3° C. than it was at 21° C.

UNIVERSITY OF WYOMING
LARAMIE, WYOMING

LITERATURE CITED

1. ANGERER, C. A. The effect of salts of heavy metals on protoplasm. I. The action of cupric chloride on the viscosity of sea urchin eggs. Jour. Cellular and Comp. Physiol. **10**: 183–197. 1937.
2. BRENCHLEY, W. E. The essential nature of certain minor elements for plant nutrition. Bot. Rev. **2**: 173–196. 1936.
3. CHATTERJI, U. N. The effect of alcohol on the respiratory rate of leaves. Proc. Nat. Acad. Sci. India **8**: 18–28. 1938.
4. COVILLE, F. V. The influence of cold in stimulating the growth of plants. Proc. Nat. Acad. Sci. (U.S.A.) **6**: 434–435. 1920.
5. DAUGHERTY, K. The action of anesthetics on Amoeba protoplasm. Physiol. Zool. **10**: 473–483. 1937.
6. DAVIES, P. A. Irreversible injury and CO_2 production from cells of *Nitella flexilis*. Bot. Gaz. **87**: 660–664. 1929.
7. DENNY, F. E. Direct versus indirect effects upon potato amylase by chemicals which induce sprouting of dormant tubers. Contr. Boyce Thompson Inst. **4**: 53–63. 1932.
8. ————, and MILLER, L. P. Effect of ethylene chlorhydrin vapors upon dormant lilac tissue. Contr. Boyce Thompson Inst. **4**: 513–528. 1932.
9. GUTHRIE, J. D. The effect of various chemical treatments of dormant potato tubers on the peroxidase, catalase, pH, and reducing properties of the expressed juice. Contr. Boyce Thompson Inst. **3**: 499–508. 1931.
10. HAAS, A. R. C. Effect of anesthetics upon respiration. Bot. Gaz. **67**: 377–404. 1919.
11. HEILBRUNN, L. V. Action of ether on protoplasm. Biol. Bull. **49**: 461–475. 1925.
12. ————. An outline of general physiology. Philadelphia. 1943.
13. HOWARD, W. L. Physiological changes accompanying breaking of the rest period. Missouri Agr. Expt. Sta. Res. Bull. **21**. 1915.
14. IRWIN, M. Comparative studies on respiration. VI. Increased production of carbon dioxide accompanied by decrease of acidity. Jour. Gen. Physiol. **1**: 399–403. 1919.
15. LLOYD, F. E. Some effects of narcotics on Spirogyra. Anesthesia and Analgesia **3**: 9–19. 1924.
16. MARCY, B. Effect of ethylene chlorhydrin and thiourea on Elodea and Nitella. Plant Physiol. **12**: 207–212. 1937.

17. McCool, M. M. The influence of etherization on certain enzymic activities of bulbs and tubers. Science n.s. **39**: 261. 1914.
18. Medes, G., and McClendon, J. F. Effect of anesthetics on various cell activities. Jour. Biol. Chem. **42**: 541–568. 1920.
19. Miller, L. F., Guthrie, J. D., and Denny, F. E. Induced changes in respiration rates and time relations in the changes in internal factors. Contr. Boyce Thompson Inst. **8**: 41–61. 1936.
20. Northen, H. T. Protoplasmic structure in Spirogyra. III. Effects of anesthetics on protoplasmic elasticity. Bot. Gaz. **100**: 238–244. 1938.
21. ———. Studies of protoplasmic structure in Spirogyra. IV. Effects of temperature on protoplasmic elasticity. Bot. Gaz. **100**: 619–626. 1939.
22. ———. Studies on the protoplasmic nature of stimulation and anesthesia. Cytologia **10**: 105–112. 1939.
23. ———. Relationship of dissociation of cellular proteins by auxins to growth. Bot. Gaz. **103**: 668–683. 1942.
24. Thornton, F. E. The action of sodium, potassium, calcium, and magnesium ions on the plasma gel of *Amoeba proteus* at different temperatures. Physiol. Zool. **8**: 246–254. 1935.
25. Timmel, H. Zentrifugenversuche über die Wirkung chemischer Agentien insbesondere des Kaliums, auf die Viscosität des Protoplasmas. Protoplasma **3**: 197–212. 1927.
26. Weber, F. Reversible Viscositätserhöhung des lebenden Protoplasmas bei Narkose. Ber. d. bot. Ges. **40**: 212–216. 1922.

THE SOLUBLE NITROGEN FRACTIONS OF POTATO TUBERS; THE AMIDES[1]

F. C. Steward[2] and H. E. Street

(WITH SIX FIGURES)

Of the total nitrogen present in potato tubers, approximately two-thirds is in the form of compounds which are extractable by 70 per cent. alcohol. This paper deals with the nature of these soluble nitrogen compounds and more particularly with the amides. Although the amides are among the simplest substances in the soluble nitrogen fractions, complete account of them can only be rendered after somewhat laborious experiment. Furthermore these relatively simple substances, which are present in but small quantity in the tissue, command a degree of interest which is not overshadowed by the more complex nitrogenous compounds which are present in greater quantity in the cells. Indeed, the behavior of the amide-N fraction emerged as one of the outstanding features of the nitrogen metabolism of potato cells under conditions such that they possess a high degree of metabolic activity and are able to synthesize protein as well as to exhibit many other signs of vital activity—including a renewed ability to absorb and accumulate salts from dilute solutions.

Using the potato tuber as experimental material and a technique of controlling those external variables which determine the behavior of the cells, a first broad survey of the biochemistry of the tissue under conditions conducive to salt absorption has been made (**49, 50, 51**). Attention has been focused upon the inter-relations between salts, aerobic respiration, protein synthesis, and the effect on these several processes of changes in temperature, in oxygen supply, and in the nature and concentration of salts in the external solution. The synthesis of protein from the simpler nitrogen compounds is the predominant feature of the recrudescence of vital activity in these cells, and it has to be recognized that in the ultimate connection between protein synthesis, respiration, and salt absorption there must lie the clue to much that is not yet known about all these processes and, indeed, about the living system.

Hitherto the biochemical survey has relied upon indirect evidence drawn from quantitative analysis of a variety of nitrogen fractions; e.g., total nitrogen, soluble nitrogen, and the latter subdivided into amino-nitrogen and amide-nitrogen. From such data it was, however, possible to infer that the soluble nitrogen fraction of potato parenchyma contained certain amides in addition to the amino-acids which were responsible for much of the buffer capacity of the expressed sap at pH 8 to 10. Moreover, the amide groups were of two kinds: the one relatively stable, as in the case of asparagine;

[1] This is the fifth of a series of papers dealing with the biochemistry of salt absorption by plants.

[2] Temporarily attached to the Botany Department, University of Chicago.

the other, "easily hydrolyzable" as in the case of glutamine. Therefore, to understand the course of protein synthesis and the way in which the "easily hydrolyzable amide" responded to experimental conditions the more critical identification and isolation of the "easily hydrolyzable amide" was regarded (**49**) as an "essential part of future work."

It is the primary purpose of this paper to establish the identity of the amides of potato tuber and to suggest such refinements as are now known to be necessary in the methods for their indirect analysis. Ultimately it may be necessary to furnish a similar account of the other alcohol-soluble nitrogen compounds of potato tuber. Thus, bit by bit, the biochemical evidence describes the molecular machinery with which the work of the cell is carried on, just as cell cytology and the investigation of protoplasm, its membranes and vacuoles, presents a picture of the milieu in which it operates.

In a study of the nitrogenous constituents of alfalfa VICKERY (**56**) accounted for 35 per cent. of the total amide as crystalline asparagine but was unable to isolate any glutamine. This strengthened the possibility, first envisaged by SCHULZE and BOSSHARD (**47**), that amides other than asparagine and glutamine might occur in plants. VICKERY (**58**) emphasized that it has been the experience of all who have attempted to determine the chemical nature of the amides found in plants that the yield of crystalline amides (asparagine and glutamine) fell far short of the quantity to be expected from their amide nitrogen content, and he considered it probable that other amides, particularly the amides of allied amino-acids, occur in plants. Hitherto the most complete account of total amide nitrogen in terms of crystalline asparagine and glutamine is that reported by VICKERY (**59**). Crystalline asparagine was obtained in quantity from tobacco leaves equivalent to 73.9 per cent. of that in an aqueous extract of the leaves and to 92.8 per cent. of that contained in the mercuric nitrate precipitate. The corresponding figures for glutamine were 42.6 and 65.7 per cent. The possibility of the presence of another amide or amide-like substance, therefore, remained open and was, in this particular instance, strengthened by the observations of VICKERY, PUCHER, LEAVENWORTH, WAKEMAN, and NOLAN (**65**) on the nitrogenous compounds present in the stem of the tobacco plant. The possible occurrence of amides other than asparagine and glutamine lent additional interest to the examination of the easily hydrolyzable amide fraction of the potato tuber and especially so because STEWARD and PRESTON (**49**) noted that the decrease in amino-N (Van Slyke) in their "hot" alcoholic extracts as compared with their "cold" alcoholic extracts, was not identical with the decrease expected if this were due to the hydrolysis of glutamine during the preparation of the "hot" extracts.

The experimental work now reported falls into three parts. The first part consists of a critical re-examination of the analytical procedures used to estimate the soluble nitrogen fractions of the potato tuber. In the second part the isolation of the crystalline amides from potato tuber is described.

The third part deals with the separation of these amides as pure substances and their final chemical identification. A fourth section summarizes present views on the rôle of acid amides in plant metabolism.

King Edward potato tubers were used for this work. The potatoes were peeled and cylinders of medulla removed along the long axis. From these cylinders discs were cut, 2 cm. in diameter and 1 to 2 mm. in thickness. The discs, previously blotted and weighed, were either used for the immediate preparation of extracts or dried. The blotted discs were dried in a vacuum oven at 80–90° C. for one hour and then at 60° C. for 24 hours. In this investigation the protracted use of living discs under experimental conditions was not in question. Therefore, the fastidious control of disc thickness, as in the salt absorption experiments (**49**), was not practiced.

Analysis of the nitrogen fractions of potato tuber

Total nitrogen

Total nitrogen was determined by a micro-Kjeldahl-Gunning digestion with copper selenite (**5**) as catalyst. The ammonia was distilled in a Pregl micro-Kjeldahl distillation apparatus, absorbed in N/50 sulphuric acid and titrated as described under the determination of ammonia-N. The results so obtained were compared with those by use of the reduced iron method (**41**), adapted to a micro scale. Nitrate-N was determined by reduction with Devarda's alloy, using the residues from the total amide determinations. The quantity of nitrate-N present in the tubers was small, ranging from 0.03 to 0.08 mgm. N per gm. fresh tissue. The total-N by the reduced iron method exceeded that by the Kjeldahl-Gunning method by the equivalent of the nitrate-N.

Nitrogen extracted by water

The present work is concerned primarily with the simpler nitrogen fractions and only indirectly with the protein-N moiety. In the previous biochemical survey, to which reference has been made, the protein-N content of the tuber was determined as that part of the total-N which is insoluble in alcohol, and this was checked against the determination of protein-N by the trichloracetic acid method. It was shown that changes in the protein-N content which resulted from experimental treatments were the same in kind and almost identical in quantity whichever of these methods was used to determine protein-N. For convenience of extraction, therefore, the alcohol method was preferred.

Water, or more strictly the dilute solution resulting from the diluted cell sap, extracts 91 to 93 per cent. of the total-N of the fresh tissue but only 75 to 78 per cent. of the total-N of the dried tissue. In other words some part, presumably of the globulin, is "denatured" during drying and is rendered less soluble in dilute salt solution.

That part of the protein which is extracted from the dry tissue by water was made the subject of a comparison of various protein precipitants. The

techniques used were: rapid heating to 80° C., maintaining at that temperature for 10 minutes and then rapidly cooling, as recommended by VICKERY, PUCHER, CLARK, CHIBNALL, and WESTALL (**63**), and replacing the water lost during the heating as necessary; precipitation by (a) acetic acid and (b) colloidal iron as described by THOMAS (**52**); precipitation by trichloracetic acid by a method based on GREENWALD (**21**), HILLER and VAN SLYKE (**25**) and THOMAS (**52**); precipitation by cupric hydroxide after the methods of HART and BENTLEY (**23**) and of BLISH (**2**); precipitation by alcohol added to 70 per cent. concentration.

From three concordant series of comparisons the various protein precipitants could be arranged in ascending order of the nitrogen precipitated from aqueous extracts of tissue. Expressing the nitrogen precipitated as mgm. nitrogen per gm. of fresh tissue, the order is as follows: heat coagulation (0.17 gm. N per gm.) < acetic acid (0.19 mgm. N) < 0.2 or 2.5 per cent. trichloracetic acid (0.22 mgm. N) colloidal iron (0.25 mgm. N) < cupric hydroxide (0.27 mgm. N) < 70 per cent. alcohol (0.29 mgm. N) < tungstic acid (0.31 mgm. N).

The method of heating to 80° C. for 10 minutes was chosen as the general method of removing protein to avoid contaminating the extracts with other reagents as it had the added advantage that it caused no change in the amide content of the extracts and very little release of ammonia-N.

As shown by the nitrogen coagulated by heat, aqueous extracts of fresh tissue contain much more protein than extracts of dried tissue. The protein-N of various aqueous extracts made from fresh tissue during the course of this work ranged from 0.71 mgm. per gm. of tissue to 0.80 mgm. per gm. whereas the nitrogen content of the aqueous extracts of dried tissue ranged from 0.18 to 0.26 mgm. N per mg. of tissue. Therefore, the possibility is that even after heat coagulation the aqueous extracts of the fresh tissue still contained quantities of protein-N which would have been excluded by other precipitants, such as 70 per cent. alcohol. As, however, the amides, with which this work is primarily concerned, are sparingly soluble in 70 per cent. alcohol it was desirable to avoid this as solvent for the soluble-N and as precipitant for protein-N. As will become clear, there is no reason to believe that the possible presence of protein in the extracts after heat coagulation affects the determination of the amide fractions appreciably; although it does bear upon the fact that a quantitative account of the soluble-N fraction in terms of ammonia-, amide-, and amino-N is not possible on the aqueous extracts, and that the discrepancy is greater if the extract has been made from fresh rather than from dried tissue (table I). On the contrary it does become possible to account for the soluble-N in terms of ammonia-, amide-, and amino-N when the aqueous extracts (freed from heat coagulable protein) are purified, and it is immediately possible to give a satisfactory account of the alcohol-soluble nitrogen in this way (tables II, V) as STEWARD and PRESTON (**49**) showed. There is every reason, therefore, to believe that the aqueous extracts contain complex nitrogen compounds (protein or peptides) which are not coagulable by heat.

TABLE I

SOLUBLE NITROGEN FRACTIONS OF FRESH AND DRIED DISCS FROM POTATO TUBERS AS SHOWN BY ANALYSIS OF AQUEOUS EXTRACTS AT INTERVALS DURING STORAGE

EXTRACTS TUBERS STOCK 2	NITROGEN PER GM. FRESH TISSUE						N IN THE EXTRACT AFTER HEAT COAGULATION
	AMMONIA-N	EASILY HYDROLYZABLE AMIDE-N	ASPARAGINE-AMIDE-N	TOTAL AMIDE-N	AMINO-N*	NH_4 + AMINO + AMIDE-N	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Fresh tissue (E.36)	0.01	0.18	0.24	0.42	0.49	0.92	1.72
Dried tissue (E.39)	0.05	0.16	0.27	0.43	6.65	1.13	1.95
Fresh tissue (E.40)	0.001	0.18	0.27	0.45	0.74	1.19	1.75
Dried tissue (E.42)	0.03	0.21	0.32	0.53	1.1	1.66	2.11
Fresh tissue (E.46)	0.01	0.22	0.23	0.45	0.94	1.40	2.04
Dried tissue (E.47)	0.05	0.28	0.26	0.54	1.25	1.84	2.28

* Amino-N (Van Slyke) minus 80% easily hydrolyzable amide-N.

Table I shows the effect on the various nitrogen fractions of drying the tissue *before* it was extracted with water. Heat-coagulable protein was removed before determining the other fractions. Drying before extraction increases the quantity of the soluble nitrogen fractions *extracted by water;* the relative effect is greatest on the ammonia fraction though the absolute amounts involved are small. Both "easily hydrolyzable amide" and asaparagine amide fractions are but little affected by previous drying of the tissue though the true amino-fraction (Van Slyke amino-N corrected for 80 per cent. of the easily hydrolyzable amide-N) in water extracts is significantly increased by drying before extraction. Drying seems, therefore, to be accompanied by protein degradation with the release of amino-compounds soluble in water, or dilute salt solutions, but the concomitant effect of drying on the amide fractions is small.

Effect of washing the discs

The moisture content of different batches of fresh tissue used in various extractions ranged from 76.6 to 81.8 per cent.; that of tissue washed in running tap water for 24 hours ranged from 83 to 87.3 per cent. The total-N of the fresh tissue ranged from 0.26 to 0.33 per cent. of its fresh weight of which 66.7 per cent. was non-protein-N. Washing the discs in running tap water for 24 hours caused a loss of 18 to 20 per cent. of the non-protein-N, 70 per cent. of this loss being due to amino-N (Van Slyke). This confirms an earlier presumption, on indirect evidence, that one effect of washing the discs was to cause some loss of amino-acids from the cell sap (**48a**).

Comparison of aqueous and alcoholic extracts of potato tissue

Aqueous and alcoholic extracts of the fresh and dried material were prepared. The aqueous extracts were prepared by methods based on those of Chibnall (**6**), Vickery, Pucher, Clark, Chibnall, and Westall (**63**), and of Pucher and Vickery (**42**). The methods used to prepare the alcoholic extracts were essentially similar, 70 per cent. alcohol being used as solvent since it was known that stronger alcohols were likely to hinder the extraction of the simpler nitrogenous substances. The methods of extraction used did not attempt to exhaust the material. In all cases the tissue-solvent mixture was adjusted to a definite volume and then an aliquot of the filtrate taken for analysis. Extraction is therefore "complete" if the concentration of the fractions concerned is the same in the aliquots as in the tissue-solvent mixture. The following methods of extraction were adopted.

Aqueous extracts of the fresh tissue.—Twenty grams of fresh tissue, cut in discs, was pounded in a mortar and the juice poured off into a 100-ml. flask. The pounding was continued adding successive quantities of water to the crushed tissue which was finally transferred to the 100-ml. flask and the mortar washed out with water to adjust the total volume of the mixture to 100 ml. The mixture was shaken for ½ hour, allowed to settle, and the

upper clear liquid strained off through fine silk. This liquid was then rapidly heated to 80° C. for 10 minutes, rapidly cooled, and then filtered through paper. Five ml. of this extract were equivalent to 1 gram of fresh tissue.

AQUEOUS EXTRACTS OF THE DRIED MATERIAL.—A weighed amount of the dried material in no. 60 powder was placed in a 100-ml. flask, 70 ml. of water added, and the flask and contents stored at 5° C. for 18 hours, shaking occasionally. At the end of this period the flask and contents were allowed to reach room temperature, the volume of the contents adjusted to 100 ml. with water, stored for a further 2 hours at 5° C., shaking occasionally, and then rapidly filtered through paper. The clear filtrate was rapidly heated to 80° C. for 10 minutes, rapidly cooled, and then filtered.

These methods of preparing aqueous extracts from fresh and dried tissue were found to give consistent results for ammonia-N, if the proportion of tissue to extract did not exceed 0.4 gm. of fresh tissue to 1 ml. of extract. In preparing extracts for analysis STEWARD and PRESTON previously used much more solvent than this per gm. of tissue.

ALCOHOLIC EXTRACTS OF THE FRESH TISSUE.—Twenty grams of the fresh tissue discs were covered with 48 ml. of 95 per cent. alcohol and allowed to stand for 18 hours at 5° C., shaking occasionally. The clear, yellow, supernatant liquid was poured off into a 100-ml. flask and the tissue residue pounded with 70 per cent. alcohol in a mortar. The mortar was washed and the pounded tissue transferred to the 100-ml. flask with sufficient 70 per cent. alcohol to adjust the volume of the tissue-solvent mixture to 100 ml.; the suspension was then shaken for ½ hour and filtered.

ALCOHOLIC EXTRACTS OF THE DRIED MATERIAL.—These extracts were prepared exactly as the aqueous extracts of dried material, except that 70 per cent. alcohol was used as solvent in place of water.

The alcoholic extracts, *when compared with aqueous extracts prepared at the same time from the same stock of fresh or dried material,* gave lower values for easily hydrolyzable amide, asparagine-amide, and total-soluble-N (table II). The amides were incompletely extracted by 70 per cent. alcohol at a "concentration" of 0.1 grams of fresh tissue to 1 ml. of extract. At greater proportions of tissue to extract 70 per cent. alcohol was markedly less efficient as a solvent for the amide nitrogen fractions. Furthermore, the presence of the alcohol interfered with the subsequent estimation of the easily hydrolyzable amide-N giving a low value. This effect made it at first appear that the main loss of amide-N on extraction with 70 per cent. alcohol was due to incomplete extraction of the easily hydrolyzable amide-N portion. However, even when the alcohol was removed at 40° C. in vacuo prior to the analysis of the extracts, it is seen that both amides are incompletely extracted (table II).

It is clear, therefore, that tests for complete extraction of the amides should always be made whenever cold 70 per cent. alcohol is to be adopted as a solvent for the nitrogenous constituents of plant material.

TABLE II

NITROGEN FRACTIONS IN AQUEOUS AND ALCOHOLIC EXTRACTS OF FRESH AND DRIED DISCS FROM POTATO TUBERS

SOURCE	EXTRACT		GM. FRESH TISSUE PER ML. OF EXTRACT	NITROGEN PER GM. FRESH TISSUE					
				AMMONIA-N	EASILY HYDROLYZABLE AMIDE-N	ASPARAGINE-AMIDE-N	TOTAL AMIDE-N	AMINO-N*	TOTAL NON-COAGULABLE-N
			gm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Fresh tissue (Stock 2)	Aqueous	E.38	0.4	0.01	0.18	0.24	0.42	0.71	1.73
		E.40	0.2	0.001	0.18	0.27	0.45	0.74	1.75
	70% alcohol	E.37	0.2	0.002	0.12	0.23	0.35	0.82	1.36
		E.41	0.2	0.01	0.13	0.20	0.33	0.72	1.23
Dried tissue (Stock 1)	Aqueous	E.29	0.2	0.04	0.26	0.39	0.65	1.3	2.99
		E.30	0.2	0.05	0.25	0.40	0.65	1.2	2.96
		E.31	0.65	0.04	0.23	0.33	0.56	0.9	2.65
	70% alcohol	E.51	0.2	0.03	0.17	0.18	0.35	.....	1.59
		E.52	0.1	0.03	0.22	0.20	0.42	.....	1.75

* Amino-N (Van Slyke) minus 80% easily hydrolyzable amide-N.

Determination of ammonia nitrogen

Ammonia-N was determined by distillation in vacuo at 40° C. according to the method of Vickery, Pucher, and Leavenworth (**64**), using in each determination a quantity of extract equivalent to 2 gm. or more of fresh tissue. The ammonia was absorbed in 5 ml. of N/50 sulphuric acid. The excess acid was titrated against N/100 sodium hydroxide using 3 drops of Tashiro's indicator.[3] In each determination the ammonia was equivalent to not less than 0.2 ml. N/100 sodium hydroxide. Duplicate readings agreeing to 0.02 ml. N/100 sodium hydroxide could be obtained over the whole range (0.014–2.8 mgm. N) used in the trials. Using this method the fresh tissue was found to contain 0.001–0.016 mgm. ammonia-N per gm.; the dried material contained 0.03–0.08 mgm. ammonia-N per gm. fresh tissue. Similar values were obtained using an aeration apparatus as described by Woolf (**72**). Distillation in the Pregl micro-Kjeldahl distillation apparatus gave higher values for the ammonia-N.

The buffered alkaline mixture of Pucher, Vickery, and Leavenworth or an excess of magnesium oxide produced distillation mixtures of pH 9.68 to 9.74 and these gave, under the same distillation conditions, similar values for ammonia-N. Excess of calcium oxide gave a distillation mixture of pH about 12.3, and this increased the apparent ammonia-N content (table III).

To reveal the source of the increase of ammonia-N resulting from distillation of aqueous extracts in the micro-Kjeldahl apparatus as compared with distillation under reduced pressure at 40° C., the extracts were analyzed subsequent to the ammonia determinations. The higher values for ammonia-N were correlated with lower values for easily hydrolyzable amide and asparagine-amide-N. Although the absolute increase in ammonia-N consequent upon distillation in the micro-Kjeldahl apparatus was small it could be accounted for to the extent of 80 per cent. by a concomitant decrease in amide-N, and, as this was also associated with some loss of amino-N (Van Slyke), it was evident that the glutamine-like amide was being affected by these distillation conditions.

Determination of total amide nitrogen

The method used for the determination of total amide-N was based on that of Vickery, Pucher, and Leavenworth (**64**). The hydrolysis was conducted in the hydrolyzing tubes described later under the method for "easily hydrolyzable amide-N." The ammonia was recovered by distillation at 40° C. in vacuo as described in the previous section.

The content of asparagine amide-N was determined as the difference between total amide-N and the "easily hydrolyzable amide-N." The content of asparagine amide-N so calculated will represent the free asparagine present in the tissues provided that no polypeptides, containing amide groups, are present in the extracts and suffer hydrolysis under the conditions employed.

[3] One hundred ml. of 0.02 per cent. methyl red in 50 per cent. alcohol plus 15 ml. of 0.1 per cent. methylene blue in water.

TABLE III

COMPARISON OF VARIOUS PROCEDURES FOR THE DETERMINATION OF AMMONIA
(AMMONIA-N AS MGM. PER GM. FRESH TISSUE)

METHOD OF AMMONIA RECOVERY	DISTILLATION FOR 10 MINUTES IN MICRO-KJELDAHL APPARATUS		DISTILLATION FOR 10 MINUTES AT 40° IN VACUO			AERATION
ALKALI USED	MAGNESIUM OXIDE	BUFFERED ALKALINE MIXTURE	MAGNESIUM OXIDE	CALCIUM HYDROXIDE	BUFFERED ALKALINE MIXTURE	MAGNESIUM OXIDE
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
E.53	0.072	0.101	0.050	0.057	0.050	0.048
E.54	0.07	0.087	0.042	0.049	0.041	0.040
E.31	0.069	0.088	0.038	0.042	0.037	0.037
E.33	.....	.....	0.048	0.051	0.047	0.045

The tissue contained from 0.42 to 0.66 mgm. total amide-N per gram fresh tissue and 0.23 to 0.40 mgm. asparagine amide-N per gram of fresh tissue.

Determination of easily hydrolyzable amide nitrogen

The tissue extracts were submitted to the mild hydrolysis employed by Vickery, Pucher, Clark, Chibnall, and Westall (**63**) for the estimation of glutamine. The hydrolysis was performed in 25 × 200 mm. Pyrex thick-walled test tubes, closed with a rubber stopper carrying an air condenser constructed of 30 inches of glass tubing of 4 mm. internal diameter. The ammonia was recovered by vacuum distillation as described above under the determination of ammonia nitrogen.

The fresh tissue contained 0.15–0.28 mgm. easily hydrolyzable amide-N per gram of fresh tissue (average value 0.23 mgm. N per gram fresh tissue).

This standard method for the determination of easily hydrolyzable amide-N was modified in various ways to see how far the conditions of hydrolysis of the "easily hydrolyzable amides" of potato tissue were identical with those of glutamine.

After 2 hours' hydrolysis at pH 6.5, 99.7 per cent. hydrolysis of glutamine has occurred, and hydrolysis was complete in 2 hours; i.e., the increase in glutamine amide-N on prolonging the hydrolysis from 2 to 3 hours was only 0.3 per cent. A glutamine-asparagine solution was prepared with an amide content comparable to that of the potato extracts. If the hydrolysis of this solution was prolonged from 2 to 3 and 4 hours, the increases obtained were 1.4 and 3.0 per cent., respectively. Crystalline mixtures (45.III) were selected because they contained the easily hydrolyzable amides and asparagine-amide in similar proportions to that in the tissue. The yield of easily hydrolyzable amide-N was increased by prolonging the hydrolysis from 2.0 to 3.0 and 4.0 hours by only 2.0 and 3.8 per cent., respectively. Solutions obtained by liberating the nitrogen compounds from mercuric nitrate precipitates responded to hydrolysis prolonged from 2.0 to 3.0 hours by an increase of 2.9 and 4.0 per cent., respectively, in the yield of easily hydrolyzable amide-N. The easily hydrolyzable amide nitrogen of various aqueous extracts of potato tuber tissue increased with the period of hydrolysis from two hours to three and four hours by increases of 6.0 and 8.5 per cent., respectively, in the amide as so determined. Clearly the more purified the extracts become, the more closely does the time required to hydrolyze the easily hydrolyzable amide resemble that expected from glutamine in contact only with asparagine.

The hydrolysis of the easily hydrolyzable amides of potato tissue and of glutamine were both retarded by alcohol to a similar extent.

If the concentration of alcohol in the hydrolyzing solution was about 23 per cent. (as when using extracts containing 70 per cent. alcohol) the hydrolysis after 2 hours' heating had proceeded to the extent of 86 per cent. in the case of a solution of pure glutamine; whereas for the easily hydrolyzable amides in the tissue extracts it had proceeded to the extent of 71 to 80 per cent.

Therefore, for the analysis of amides the alcohol must be removed from the extracts, under conditions which do not cause decomposition of the unstable amide fraction; otherwise low values for easily hydrolyzable amide-N and correspondingly high values for asparagine amide-N will be obtained.

Determination of amino nitrogen

Amino-N was determined in the Van Slyke micro-amino-apparatus (**53, 54**) using a reaction time of 10 minutes. Extract yielding 0.7–2.0 cc. of nitrogen gas, freed from ammonia if necessary and acidified with acetic acid, was used for each test. If undue frothing occurred when the reaction chamber was shaken, 1 drop of toluene was added to the extract before it was drawn into the reaction-chamber. As recommended by the Committee on Methods of Analysis of the American Society of Plant Physiologists (**1**), alcohol was removed from the alcoholic extracts by distillation in vacuo at 40° C. prior to the determination of the amino-N. The value for amino-N obtained by this method is referred to as "amino-N (Van Slyke)."

When the amino-N (Van Slyke) of a solution of glutamine was determined, it was found that 91.9 per cent. of its total-N was evolved as nitrogen gas in 10 minutes. This corresponded to the conversion to nitrogen of 84 per cent. of the amide group. When glutamine is submitted to hydrolysis at pH 6.5, under the conditions here used for the determination of easily hydrolyzable amide-N, it yields quantitatively ammonia and pyrrolidone-carboxylic acid, so that complete loss of amino-N (Van Slyke) occurs (**42, 63**). Therefore, upon hydrolysis of glutamine at pH 6.5, and as shown by Vickery, Chibnall *et al.* (**63**), the fraction

$$\frac{\text{decrease in amino-N (Van Slyke) as a result of amide hydrolysis}}{\text{easily hydrolyzable amide-N content}}$$

has a value of 1.8. This value should be obtained with plant extracts containing glutamine, provided that no other substances are present which produce ammonia or change in amino-N (Van Slyke) content under the conditions of glutamine hydrolysis. Lower values than 1.8 would be expected with compounds such as allantoin and urea (**39, 63**) or unstable peptides of the type prepared by Melville (**34**), which resemble glutamine in their stability and give, though not so markedly, high values in the Van Slyke amino-N determination. Thus, in presence of glutamine, the true amino-N calculated as amino-N (Van Slyke) minus 80 per cent. glutamine amide-N.

The decrease in amino-N (Van Slyke) under the conditions of glutamine hydrolysis is additional qualitative evidence of the presence of this amide and serves to distinguish it from other substances such as urea and allantoin which give rise to ammonia on mild hydrolysis (**8, 63**). In the cases examined the ratio varied from 1.47 to 1.77 (table IV). This result is consistent with the identity of the easily hydrolyzable amide with glutamine although in the work of Steward and Preston (**49**), the amino-groups seemed too

stable under conditions of amide hydrolysis for this hypothesis to be accepted. In the earlier work amide-N was determined by the increase in ammonia-N in "hot" as compared with "cold" alcohol extracts, and it now seems probable that under these conditions of amide hydrolysis the glutamine yielded glutamic acid, wholly or in part, rather than pyrrolidone carboxylic acid.

The amino-N (Van Slyke) value was normally determined on extracts from which ammonia had been removed under conditions which did not cause hydrolysis of the "easily hydrolyzable amides." If, therefore, the easily hydrolyzable amide fractions consist entirely or mainly of glutamine then the amino-N (Van Slyke) value will exceed the true amino-N by an amount equal to 80 per cent. of the easily hydrolyzable amide-N.

Table V shows a group of analyses in which the sum of the amino-N (Van Slyke), total amide-N, and ammonia-N, and this sum minus 80 per

TABLE IV

THE EFFECT OF AMIDE HYDROLYSIS ON THE VALUE FOR AMINO-N (VAN SLYKE)

Extract	Nitrogen per gm. fresh tissue				Loss of amino-N (Van Slyke) as a fraction of easily hydrolyzable amide-N
	Easily hydrolyzable amide-N	Amino-N (Van Slyke)			
		After removal of ammonia-N	After hydrolysis of the easily hydrolyzable amides	After hydrolysis of the total amides	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
E.29	0.26	1.54	1.07	1.29	1.69
E.30	0.25	1.22	0.85	1.2	1.48
E.42	0.21	1.27	0.93	.....	1.60
E.53	0.28	1.51	0.01	1.13	1.77
E.54	0.23	1.31	0.97	1.15	1.47

cent. of the easily hydrolyzable amide-N, is expressed as a percentage of the total-N in the extracts after removing any heat coagulum. The sum of amino-N (Van Slyke), total amide-N, and ammonia-N does not account fully for the total soluble-N of the aqueous extracts but tends to exceed it in the purer extracts, the excess increasing in the series: purified aqueous extracts < alcoholic extracts < the crystalline mixtures in ascending order of purity. (These mixtures were obtained in the work on the isolation of the amides later described herein.) When, however, the value for amino-N (Van Slyke) is reduced by an amount equal to 80 per cent. of the easily hydrolyzable amide-N the sum of *true* amino, amide, and ammonia-N expressed as a percentage of the total non-protein-N increases through the series: aqueous extracts < purified aqueous extracts < alcohol extracts < crystallizations from the original mother-liquor < re-crystallized mixtures. The total of the soluble nitrogen fractions thus analyzed never significantly exceeded the total soluble-N of the extracts although it gradually approached this as purification proceeded until, in the purest crystalline mix-

tures examined, an almost exact account of the total-N was rendered. The account, by the means described, of the soluble nitrogen of the alcoholic extracts of the tissue tended to be 7 per cent. too low (average of 3), or ignoring one low result, only about 2 per cent. too low. STEWARD and PRESTON (**49**) obtained evidence that the amide group of the easily hydrolyzable amide fraction reacted to the extent of 79–92 per cent. in the Van Slyke determination. The evidence here presented amply confirms the reactivity of this amide group in the Van Slyke determination and shows that the behavior of the tissue extracts is in close accord with that of a pure solution of glutamine in this respect; i.e., 80 per cent. of amide group reacts in Van Slyke determination.

The true amino-N is, therefore, best estimated by correcting the Van Slyke amino-N on this basis. The true amino-N (amino-N (Van Slyke)—80 per cent. easily hydrolyzable amide-N) for all the samples of fresh tuber tissue which were examined fell within the limits 0.7 to 1.05 mgm. amino-N per gm. The dried tissue contained 1.1 to 1.35 mgm. true amino-N per gm. fresh tissue. The true amino-N, as thus defined, includes the amino-N groups of the amino acids of both stable and easily hydrolyzable amide fractions.

If the amino-N (Van Slyke) was determined on aqueous extracts after the acid hydrolysis used in the determination of total amide-N, the values so obtained were lower than those obtained on the original extracts freed from ammonia, but they were higher than the results obtained after the mild hydrolysis used in the determination of the easily hydrolyzable amide-N (table V). This may be due to hydrolysis of peptides during the total amide determination or, if the easily hydrolyzable amides are glutamine, to inhibition of pyrrolidone carboxylic acid formation in strongly acid solution (**18**).

Isolation of crystalline amides from potato tuber

Present methods for the isolation of acid amides from plant tissues are based on the pioneer researches of E. SCHULZE and his co-workers. SCHULZE employed lead acetate to clarify the plant extract and mercuric nitrate (**45**) to precipitate the amides. NEUBERG and KERB (**37**) in 1912 used mercuric acetate as an alternative precipitant.

Glutamine was isolated by SCHULZE and BOSSHARD (**46**) in 1883 by treating the fresh sap of the beetroot with lead acetate and then treating the filtrate with a neutral solution of mercuric nitrate. VICKERY, PUCHER, and CLARK (**61**), using the same method, obtained a quantitative yield of glutamine. EISENCHIMMEL (**16**) employed the NEUBERG and KERB reagent (**37**) to isolate glutamine from beetroot juice.

PRECIPITATION OF NITROGEN FRACTIONS OF POTATO EXTRACTS WITH VARIOUS REAGENTS

Fractionations by procedures based on those of VICKERY, PUCHER, and CLARK (**61**) and of VICKERY (**57**) were carried out to develop a method suit-

TABLE V

NITROGEN IN EXTRACTS IN TERMS OF AMMONIA-N, AMIDE-N, AND AMINO-N

	NITROGEN PER GM. FRESH TISSUE					SUM OF AMMONIA-, AMIDE-, AND AMINO-N (VAN SLYKE) AS PERCENTAGE OF THE TOTAL-N IN EXTRACTS*	SUM OF THE AMMONIA-, AMIDE-, AND AMINO-N (VAN SLYKE) MINUS 80% OF THE EASILY HYDROLYZABLE AMIDE-N AS A PERCENTAGE OF THE TOTAL-N IN EXTRACTS*
	AMMONIA-N	EASILY HYDROLYZABLE AMIDE-N	TOTAL AMIDE-N	AMINO-N (VAN SLYKE)	TOTAL-N IN EXTRACTS		
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	%	%
Aqueous extracts of fresh tissue							
E.34	0.016	0.26	0.60	1.22	2.30	80	71
E.40	0.001	0.18	0.45	0.88	1.75	76	68
E.46	0.009	0.22	0.45	1.12	2.04	77	69
Aqueous extracts of dried tissue							
E.29	0.04	0.26	0.65	1.54	2.79	80	72
E.32	0.04	0.23	0.61	1.36	2.50	80	73
E.42	0.03	0.21	0.53	1.27	2.11	87	79
E.47	0.05	0.28	0.54	1.47	2.28	90	81
Purified aqueous extracts							
E.31.D	0.04	0.21	0.48	0.97	1.61	92	83
E.32.D	0.016	0.20	0.45	1.03	1.72	87	77
E.32.E	0.001	0.19	0.41	0.88	1.26	102	90
E.45.D	0.49†	0.18	0.35	0.84	1.68	100	91
E.46.D	0.35†	0.16	0.37	0.74	1.46	100	93
70% alcohol extracts							
E.41	0.01	0.16	0.37	0.96	1.23	109	99
E.43	0.02	0.14	0.29	0.83	1.23	93	84
E.44	0.02	0.17	0.35	1.0	1.31	104	95

* After removal of heat coagulum, if any.
† Neutralized with ammonia.

TABLE V—(*Continued*)

	Nitrogen per 100 mgm. crystalline material					Sum of ammonia-, amide-, and amino-N (Van Slyke) as percentage of the total-N in extracts*	Sum of the ammonia-, amide-, and amino-N (Van Slyke) minus 80% of the easily hydrolyzable amide-N as percentage of the total-N in extracts*
	Ammonia-N	Easily hydrolyzable amide-N	Total amide-N	Amino-N (Van Slyke)	Total-N in extracts		
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	%	%
First crystallizations from mother liquor							
46.iii	.....	1.58	8.26	10.5	18.38	102	95
46.iv	.....	5.11	8.19	12.7	18.53	113	90
46.v	0.29	3.74	7.01	10.8	16.9	107	89
46.vi	0.64	4.61	5.58	11.4	14.9	118	93
46.viii	0.42	3.70	5.84	11.0	15.85	109	91
Recrystallized material							
45.iii.1	.....	3.65	7.7	12.3	17.12	117	100
45.iii.3	.....	6.08	7.84	13.1	16.7	125	96
45.iii.3a	.....	6.17	8.1	12.9	17.3	121	99
45.iii.3b	.....	5.5	7.54	12.6	16.6	127	100
45.iii.1a	.....	3.75	5.54	10.95	13.46	122	100
45.iii.1b	.....	4.91	7.69	14.9	19.3	117	97
45.iii.1c	.....	2.73	8.54	11.4	17.7	113	100

able for the isolation of the easily hydrolyzable amides and to see how far they behaved like glutamine to the precipitant used. These experiments showed (table VI):

1. The easily hydrolyzable amide-N survives lead acetate precipitation and is almost quantitatively removed by mercuric nitrate reagent; and on transfer from the mercuric nitrate precipitate, it largely escapes subsequent precipitation with phosphotungstic acid. The yield of this fraction as precipitated by the Neuberg and Kerb reagent is not as good as by mercuric nitrate.

TABLE VI

PERCENTAGE RECOVERY OF THE VARIOUS NITROGEN FRACTIONS IN PURIFIED EXTRACTS OF POTATO TUBER

NITROGEN FRACTION IN THE EXTRACT	RECOVERY OF THE NITROGEN FRACTIONS: NITROGEN FRACTION IN THE PURIFIED EXTRACT AS PERCENTAGE OF THAT SAME FRACTION IN THE ORIGINAL AQUEOUS EXTRACT OF THE TUBERS				
	EXTRACT CLARIFIED BY TREATMENT WITH EXCESS LEAD ACETATE REAGENT		EXTRACTS PREPARED BY DECOMPOSITION OF THE MERCURY COMPOUNDS PRECIPITATED FROM AQUEOUS EXTRACTS CLEARED BY LEAD ACETATE		EXTRACTS PREPARED FROM PHOSPHOTUNGSTIC ACID PRECIPITATES FROM EXTRACTS OBTAINED FROM PRECIPITATED MERCURY COMPOUNDS
	E.31	E.32	MERCURIC NITRATE REAGENT E.31	NEUBERG AND KERB REAGENT E.32	
	%	%	%	%	%
Ammonia-N	100	112	100	40	2.5
Easily hydrolyzable amide-N	92	97	89	86	81
Asparagine-amide-N	93	87.5	85	73	65
Amino-N*	97	96	91.5	83	62
Sum of ammonia-, amide-, and amino-N	95	95	90	80	64
Total soluble-N extracted†	93	93	65	69	50

* Van Slyke amino-N minus 80% of amide-N.
† Excluding heat coagulum.

2. The asparagine amide-N survives lead acetate precipitation, is almost quantitatively removed by mercuric nitrate (but less so by Neuberg and Kerb reagent), and is much more liable to reprecipitation by phosphotungstic acid.

3. The true amino-N of aqueous extracts, which also survives lead acetate precipitation, is removed almost quantitatively by mercuric nitrate (less so by Neuberg and Kerb's reagent) and is reprecipitated by phosphotungstic acid to approximately the same degree as the asparagine amide-N.

4. That part of the total-N of the aqueous extracts for which no account has yet been rendered up to this point (nitrogen other than heat-coagulated

nitrogen and ammonia-, amino-, and amide-N) escapes lead acetate precipitation and is precipitated less readily by mercuric nitrate and more readily by phosphotungstic acid than the other nitrogen fractions. While the behavior toward lead acetate rather precludes this being stable protein it must be some more complex fraction than the others and is probably rich in diamino-acids; this warrants further investigation beyond the scope of this paper. Therefore the basis of the quantitative isolation of the mixed amides of potato was:

1. Precipitation of aqueous extract by lead acetate.
2. Precipitation by mercuric nitrate from the lead-free filtrate.
3. Crystallization from the solution obtained on liberation of the nitrogen fractions from the mercury precipitate.

The product so obtained must contain both asparagine and easily hydrolyzable amide as well as some amino-N other than that accounted for by asparagine or glutamine. From this point the isolation of pure products depends on fractional crystallization. This procedure was put into effect on a relatively large scale as follows.

Fractionation of the nitrogen in the expressed sap of potato tubers (large scale)

The expressed juice was fractionated as described below. Prior to expression of the juice the tubers were washed, peeled, wiped with a dry cloth, cut into thin discs which were blotted, and immediately weighed. Two batches of 2.5 kgm. (extract 45) and 3.6 kgm. (extract 46) were used. The sequence of operations as indicated by the treatment of 3.6 kgm. in preparation of E.46 was as follows.

Preparation of extract "A".—Three and six-tenths kgm. of the fresh tubers were minced and the sap expressed in a double-action tincture-press. From the minced marc a second portion of sap was expressed and a third portion after remincing the marc with washed, dried, silver sand. The total yield was 2050 ml. and since the moisture content of the original fresh tissue was 81.3 per cent. of the fresh weight, the sap so expressed was equivalent to the nitrogen fractions of 2521 gm. of tissue. Fifty ml. of this liquid was removed for the analysis of extract A.

Preparation of extract "B".—The remainder of extract A was immediately heated to 75° C. for 10 minutes, rapidly cooled, and 2140 ml. of clear filtrate were collected; 60 ml. were removed for the analysis of Extract B.

Preparation of extract "C".—The remainder of extract B was treated with a slight excess (275 ml.) of lead acetate reagent. The precipitate was removed by filtration, washed on the filter with boiled and cooled distilled water, and then rejected. The combined filtrate and washings (2275 ml.) constituted extract C, of which 50 ml. was removed for analysis. The aliquot of extract C removed for analysis was treated with hydrogen sulphide, precipitated lead sulphide removed by filtration, and the filtrate and

washings freed from hydrogen sulphide by distillation at 40° C. in vacuo. The residue was diluted to 50 ml. with water and was ready for the analysis of extract C.

PREPARATION OF EXTRACT "D".—The remainder of extract C was treated with a slight excess (250 ml.) of mercuric nitrate reagent, neutralized to litmus by addition of 20 per cent. w/v solution of sodium hydroxide and set aside overnight in the refrigerator. A white precipitate settled, leaving a clear supernatant liquor which was decanted and passed through the filter. The precipitate was suspended in 500 ml. of distilled water, shaken and allowed to stand, and the supernatant liquor again removed. The precipitate collected on a hard paper was washed with distilled water, the washings being rejected. To the mercuric nitrate precipitate suspended in 1000 ml. of distilled water 1 ml. of 1/N sulphuric acid was added and the suspension treated with hydrogen sulphide. After removal of the filtered and washed mercuric sulphide the combined filtrate and washings were freed from excess hydrogen sulphide by distillation at 40° C. in vacuo for 20 minutes. The solution was then neutralized to litmus with dilute ammonia (14 ml.) From the resultant neutral solution (1300 ml.) 50 ml. was removed for analysis of extract D.

PREPARATION OF CRYSTALLINE PRODUCTS FROM EXTRACT "D".—The remainder (1250 ml.) of extract D was concentrated by distillation to 400 ml. and freed from the last traces of mercuric sulphide by filtration through paper pulp. The combined filtrate and washings were then reduced to about 190 ml. and set aside overnight in the refrigerator. The crystalline deposit which separated (46 I.) was collected and washed with 5 ml. of boiled, cooled, distilled water. After filtrate and washings were again concentrated to about 125 ml., a further crystalline deposit (46.III) separated overnight in the refrigerator, and this was treated as described for 46.I. On concentrating the remaining solution to 60 ml. the crystalline deposit (46.III) separated. This was collected and washed with 10 ml. of 70 per cent. alcohol. From this point the crystals were washed with alcohol, hot water, and the further crystallization was carried out progressively from richer mixtures of alcohol. The filtrate and washings from 46.III were reduced to 40 ml.; crystalline deposit (46.IV) was separated overnight and washed with 10 ml. of 70 per cent. alcohol. The filtrate and washings from 46.IV were reduced to 30 ml., and 50 ml. of 80 per cent. alcohol was added with constant stirring. From the liquor the crystalline deposit (46.V) separated, and this was in turn collected and washed with 70 per cent. alcohol. To the filtrate and washings (70 ml.) was added an equal volume of 90 per cent. alcohol and on setting aside the liquid in the refrigerator crystalline deposit (46.VI) separated; this was collected and washed with 80 per cent. alcohol. The mother liquid, set aside for several days in the refrigerator, gave a further deposit (46.VII). All these crystalline deposits (46.I to 46.VII) were collected on Whatman no. 50 paper, dried in a vacuum desiccator over sulphuric acid for at least 12 hours, and weighed.

For the larger vacuum distillations referred to above a distillation apparatus similar to that described by VICKERY and PUCHER (60) was used. Concentration of the mother liquors was effected by vacuum distillation on a water bath at a temperature of 40° C.

The analysis of the nitrogen fractions of the various extracts and crystalline deposits is shown in table VII, and the yield of the various nitrogen

TABLE VII

NITROGEN ANALYSES SHOWING THE COMPOSITION OF THE EXTRACTS EQUIVALENT TO 2500 GM. OF FRESH TUBERS (EXTRACT NO. 46) AND OF THE CRYSTALLINE DEPOSITS OBTAINED (46.i–vii) DURING PROGRESSIVE PURIFICATION OF AMIDE FRACTIONS

EXTRACT 46	NITROGEN PER GM. OF FRESH TISSUE					TOTAL SOLIDS, MGM. PER GM. OF FRESH TISSUE
	AMMONIA-N	EASILY HYDROLYZABLE AMIDE-N	ASPARAGINE AMIDE-N	AMINO-N (VAN SLYKE)	TOTAL-N	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
A	.....	.....	.....	.....	2.82	39.0
B	0.009	0.22	0.23	1.12	2.04	32.1
C	0.016	0.21	0.19	0.95	1.61	25.6
D	0.353*	0.16*	0.20	0.74	1.46	9.8
CRYSTALLINE DEPOSITS 46	**NITROGEN PER 100 MGM. OF CRYSTALLINE DEPOSIT†**					**YIELD IN GM. OF CRYSTALLINE DEPOSIT**
						gm.
i†	.....	.....	.....	.....	8.1	0.2175
ii†	.....	.....	.....	.....	16.0	0.2575
iii	.....	1.58	6.68	10.5	18.38	2.515
iv	.....	5.11	3.08	12.7	18.53	3.5725
v	0.29	3.74	3.27	10.8	16.9	1.413
vi	0.64	4.61	0.97	11.4	14.9	1.565
vii	0.42	3.70	2.14	11.0	15.85	0.457

* A loss of easily hydrolyzable amide occurred here due to the fact that after adding the mercuric nitrate reagent there was an interval of 4 hours before the solution was neutralized.

† Crystalline deposit 46.i was recrystallized from water shown to consist mainly of tyrosine; crystalline deposit 46.ii was shown to consist of 53% asparagine and a small amount of tyrosine.

fractions obtained in the treatment of both extracts E.46 and E.45 are shown in table VIII.

RECOVERY OF NITROGEN FRACTIONS IN THE VARIOUS EXTRACTS AND DEPOSITS

An inadvertent loss of easily hydrolyzable amide-N from the mercuric nitrate precipitate, in the fractionation of extract 46, reduced the yield so that only 71.5 per cent. of the easily hydrolyzable amide of the fresh tissue was recovered in the crystalline deposits. This was however 98.0 per cent. of that contained in the mercuric nitrate precipitate. In the fractionation of extract E.45 this loss was not incurred, and 93 per cent. (as compared with 73 per cent. in the case of E.46) of the easily hydrolyzable amide-N of

the fresh tissue was contained in the mercuric nitrate precipitate. The yield in the crystalline deposits from E.45 was, however, less quantitative due to the adoption of a less effective method of crystallization, so that the yield of easily hydrolyzable amide-N in the crystalline mixtures fell to 70 per cent. of that in the original fresh tissue. The content of asparagine amide-N and total amide-N in the crystalline deposits also represented about 70 per cent. of the original amount in the fresh tissue. It will now be shown that the easily hydrolyzable amide can be identified as glutamine and that the

TABLE VIII

PERCENTAGE RECOVERY OF THE VARIOUS NITROGEN FRACTIONS DURING ISOLATION OF AMIDES IN LARGE-SCALE EXPERIMENTS

NITROGEN FRACTION	RECOVERY OF NITROGEN FRACTIONS: NITROGEN FRACTION IN THE EXTRACT OR DEPOSITS AS PERCENTAGE OF THAT FRACTION IN THE ORIGINAL AQUEOUS EXTRACT OF THE TUBERS						
	EXTRACT CLARIFIED BY TREATMENT WITH EXCESS LEAD ACETATE (C)		EXTRACT PREPARED BY DECOMPOSITION OF MERCURIC NITRATE PRECIPITATE (D)		CRYSTALLINE DEPOSITS		PURE SUBSTANCES
	E.45	E.46	E.45	E.46	E.45	E.46	E.46
	%	%	%	%	%	%	%
Easily hydrolyzable amide-N	97.0	95.5	93.0	73.0	65.0	71.5	66.1 (as glutamine)
Asparagine amide-N	86.5	83.0	84.0	87.0	71.5	68.3	60.9 (as asparagine)
Total amide-N	89.0	88.0	89.0	82.0	68.5	71.6	62.0
Amino-N (Van Slyke)	91.5	85.0	65.0	66.0	.....	.....	36.3 (as amides and tyrosine)
Total soluble-N extracted (excluding heat coagulum)	82.0	79.0	80.0	71.0	37.3	36.6	.....
Total-N	62.0	57.0	60.5	52.0	28.2	26.8	.....

yield of pure glutamine obtained from these crystalline deposits represented 66 per cent. of that in the original tissue. The yield of pure asparagine accounted for 61 per cent. of that in the fresh tissue. Of the total amino-N (true amino-N plus amide-N which reacts in the Van Slyke reaction) only 36 per cent. is as yet definitely accounted for (table VIII) so that the identity of the remainder of this part of the soluble-N fraction of potato tuber still presents problems.

Separation of pure amides and their chemical identification

Tyrosine (46.I) was the first substance to crystallize from the concentrated solution after decomposition of the mercuric nitrate precipitate. Subsequently mixtures (46.II) of tyrosine and asparagine separated. The separation of the tyrosine and the asparagine from these mixtures depends

upon the very slight solubility of tyrosine in cold water. The further crops of crystals from the mother liquor were mixtures rich in amide nitrogen and contained both forms of amide (46 III-VII).

The crystalline mixtures rich in amide nitrogen (46.III–VII) were submitted to further crystallizations in an endeavor to isolate quantitatively the easily hydrolyzable amides. Mixtures rich in asparagine were repeatedly recrystallized in order to recover any easily hydrolyzable amide-N they contained, whereas the fractions rich in easily hydrolyzable amide were submitted to the minimum number of recrystallizations.

The crystalline material from extract 45 was used for the identification of glutamic acid (isolated as the hydrochloride) and of aspartic acid (isolated as copper aspartate) in the products of amide hydrolysis, and also for a study of the conditions under which the amides could be separated by crystallization from aqueous and aqueous-alcoholic mother-liquors using the more ready crystallizing of the stable amides from water and the relative insolubility of the easily hydrolyzable amides in alcohol. By repeated fractional crystallization, pure samples of tyrosine, asparagine, and glutamine were obtained from the crystalline deposits from E.45. As shown in table V, the methods of analysis give a complete account of the nitrogen of these fractions in terms of easily hydrolyzable amide, asparagine amide, and true amino-N. Data so obtained formed the basis of the treatment of the crystalline deposits from extract 46.[4]

The properties on which the separation of asparagine and glutamine depends and the way in which these were applied to isolate pure glutamine as the easily hydrolyzable amide and pure asparagine as the stable amide are indicated below:

(1) Asparagine is less soluble in water than glutamine. Glutamine is soluble 1 in 28 at 18° C.; asparagine 1 in 47 at 20° C. (**2**). The solubility of both is increased with rise in temperature. Glutamine, however, may suffer decomposition if heated above 60° C. in aqueous solution.

(2) Asparagine and glutamine do not form mixed crystals (**70**). Asparagine crystallizes readily from aqueous solution in the presence of impurities, whereas glutamine does not (**13, 14**). As the purity of the solutions increases by repeated fractional crystallization, glutamine crystallizes more readily.

(3) Both amides are sparingly soluble in alcohol. The solubility of asparagine in 50 per cent. alcohol at 9° C. is about 0.08 per cent. The lower solubility of asparagine accounts for the fact that addition of alcohol (up to 50 per cent. concentration) to a saturated solution of glutamine and asparagine will precipitate a mixture containing a higher proportion of asparagine than the mother liquor.

(4) The crystalline mixtures of the amides obtained from potato con-

[4] At this point in the investigation valuable advice on the technique of isolating glutamine from admixture with asparagine was received from DR. G. W. PUCHER of the Connecticut Agricultural Experiment Station.

tained a "soluble impurity" not included in the amide fraction. Mixtures rich in glutamine but containing a trace of asparagine and of this "soluble impurity" could be purified by extraction with the minimum amount of water necessary to dissolve out these impurities.

(5) The purest preparations obtainable by fractional crystallization could be further purified by treatment with Norit (activated charcoal).

The treatment of the mixtures rich in amide-N, based on the above data, was as follows: the crystalline deposit was dissolved in the minimum amount of water at 60° C. and then set aside in the refrigerator at 8–10° C. Asparagine, or a mixture very rich in asparagine, crystallized out. The filtrate was then treated with not more than an equal volume of alcohol (the amount used being adjusted according to the nature of the crystalline preparation used) and set aside in the refrigerator. Asparagine or a mixture of asparagine and glutamine crystallized out. The filtrate was reduced to a small volume in vacuo at low temperature and then treated with an equal volume of alcohol, chilled, and then treated with a second volume of alcohol. The mother liquor, on storage overnight in the refrigerator at 5° C., gave either crystalline glutamine or a mixture rich in glutamine.

This standard procedure was applied to all of the amide-rich mixtures, and the crops of crystals were grouped into four categories: (1) asparagine; (2) mixtures of asparagine and glutamine which often consisted of spherical masses of glutamine adhering to the large transparent crystals of asparagine (figs. 1, 2); (3) glutamine containing a detectable trace of asparagine; and (4) glutamine free from asparagine. Category 2 was gradually eliminated by repeated applications of the standard procedure. As the crystalline mixtures became purer no sharp separation of glutamine from asparagine was possible, as the glutamine showed an increasing tendency to crystallize out readily. Glutamine-rich fractions were therefore obtained which contained a trace of asparagine and an impurity very soluble in water. To remove both of these impurities the glutamine-rich fractions were treated with a small amount of water. The undissolved residues were crude glutamine. The filtrates were submitted to fractional crystallization until glutamine-rich fractions were again obtained. The identity of tyrosine, asparagine, and glutamine obtained is established by the following data.

Tyrosine

The crystalline mixtures (fig. 1) composed of tyrosine and asparagine (46.I,II) yielded by fractional crystallization 0.288 gm. tyrosine and 0.12 gm. asparagine. The tyrosine had a melting point (with decomposition) of 256–258° C. (uncorr.). The elementary analysis found C, 58.6 per cent.; H, 6.14 per cent.; N, 7.6 per cent. Tyrosine requires C, 59.6 per cent.; H, 6.12 per cent.; N, 7.73 per cent.

Asparagine

The crystalline mixtures rich in amide nitrogen yielded 2.66 gm. of asparagine. This substance had a melting point (sealed tube, with decom-

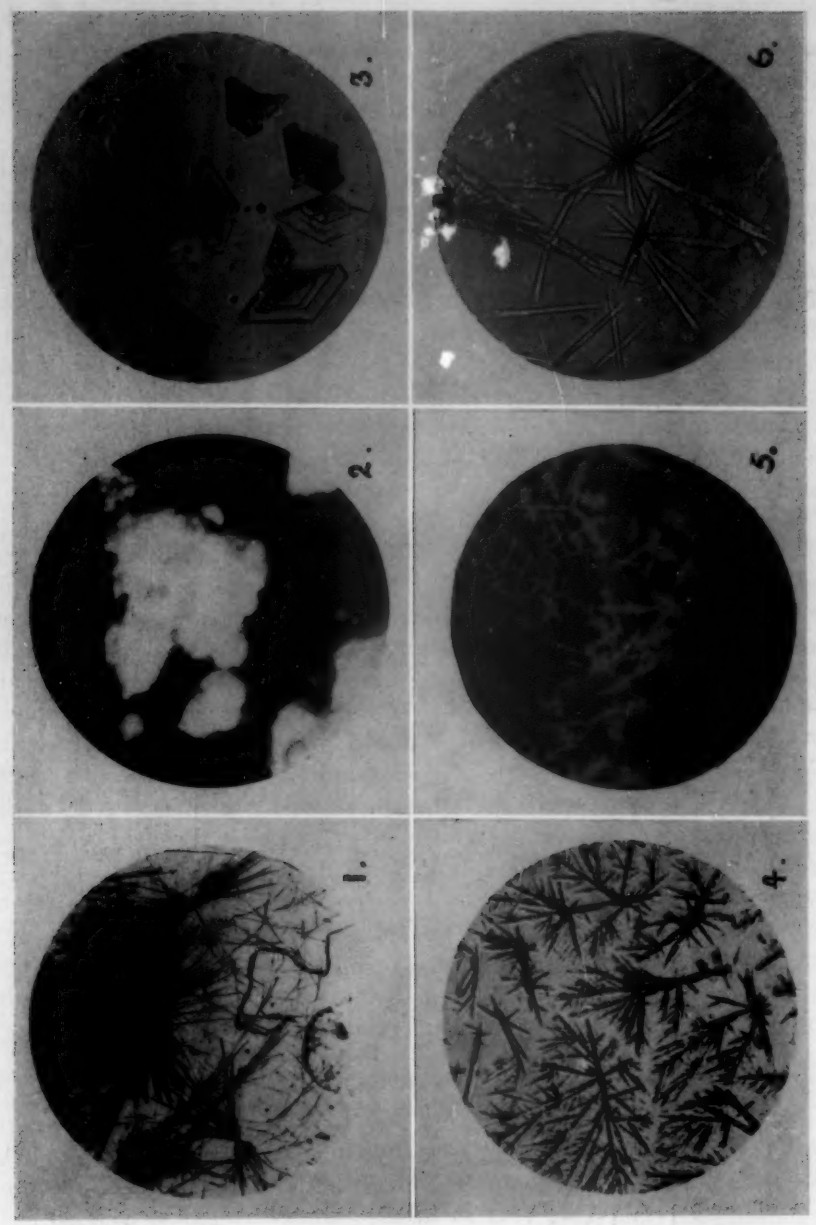


FIG. 1. Tyrosine (×30). FIG. 2. Mixture of asparagine and glutamine (×44). FIG. 3. Asparagine (×70). FIG. 4. Crude glutamine (×48). FIG. 5. Glutamine (×55). FIG. 6. Glutamine (×175). (Photographs by A. Bates.)

position) of 226° C. (uncorr.). The nitrogen analysis found N, 18.3 per cent.; asparagine-amide-N, 9.1 per cent.; and amino-N (Van Slyke), 9.08 per cent.; the amide hydrolyzed under the conditions of hydrolysis of the easily hydrolyzable amide was only 2.6 per cent. of the total.

This sample of asparagine, dissolved in the minimum amount of water at 70° C. and treated with 100 mgm. Norit for 10 minutes at 70° C. yielded 2.44 gm. of *recrystallized asparagine*. The recrystallized asparagine had a melting point (sealed tube, with decomposition) of 227° C. (uncorr.). The elementary analysis found C, 32.2 per cent.; H, 6.69 per cent.; N, 18.6 per cent.; asparagine-amide-N, 9.3 per cent.; and water of crystallization 12.0 per cent. The identity of pure asparagine is therefore established.

Glutamine

The amount of crude glutamine obtained was 3.30 gm. (fig. 4). This had a melting point of 182° C. (uncorr.) and a mixed m.pt. of 181° C. with a sample of pure glutamine at m.pt. of 182° C. The nitrogen analysis found N, 18.7 per cent.; glutamine amide-N, 8.7 per cent.; and amino-N (Van Slyke), 15.2 per cent.; 3.05 gm. of glutamine purified with Norit were obtained (figs. 5, 6). This had a melting point of 184° C. (uncorr.). The elementary analysis of the purified glutamine found C, 39.9 per cent.; H, 6.86 per cent.; N, 19.3 per cent.; amide-N, 9.2 per cent.; and amino-N (Van Slyke), 16.5 per cent. Glutamine requires C, 41.1 per cent.; H, 6.9 per cent.; N, 19.2 per cent.; and amide-N, 9.6 per cent. The optical rotation found was $[\alpha]^{7-5} = +5°$. One recrystallization from water yielded 2.71 gm. of recrystallized glutamine with a melting point of 185°C. (uncorr.). The elementary analysis of recrystallized glutamine found C, 40.5 per cent.; H, 6.78 per cent.; N, 19.1 per cent.; amide-N, 9.5 per cent.; and amino-N (Van Slyke), 17.5 per cent. The identity of glutamine as the easily hydrolyzable amide of potato tuber is, therefore, established.

The yields obtained were tyrosine, 0.116; asparagine, 1.42; and glutamine, 1.51 gm. per kgm. of fresh tissue and, as shown in table VIII, the yield of glutamine and asparagine accounts for 66 and 61 per cent., respectively, of the easily hydrolyzable and stable amide-N, present in the fresh tissue.

Vauquelin (**53**) and subsequently Hirsch and Ludwig (**26**) demonstrated the presence of asparagine in the potato. Hungerbuhler (**28**) in 1885 recorded that the mother liquor from a first crystallization of asparagine, when evaporated to dryness, yielded a residue which was not definitely crystalline. This residue, once recrystallized from water, gave on saturating with cupric hydroxide a sky-blue liquid from which a copper compound could be separated. Hungerbuhler suggested from the percentage of copper (18.4) that glutamine copper was probably present. Neuberger and Sauger (**38**) reported, during the course of the present investigation, the isolation, but in very low yield (90 mgm. per liter of expressed sap), of a specimen of glutamine (m.pt. 182° C., N content 18.65 per cent.) from King Edward potato tubers. From an extract of Majestic potato tubers they

were even unable to isolate any glutamine, although their indirect analysis indicated the presence of 1.2 gm. The yield of 1.51 gm. of crystalline glutamine per kgm. of fresh tuber tissue, here recorded, represents an isolation of 66.1 per cent. of that indicated by indirect analysis of the fresh juice and 90.5 per cent. of that contained in the mercuric nitrate precipitate. This recovery of glutamine, in the presence of a greater quantity of asparagine, represents an advance on the best results hitherto recorded (**59**). The technique of isolation from the mercuric nitrate precipitate has achieved a particularly high yield. An accidental loss of glutamine in the preparation of the mercuric nitrate precipitate obscures somewhat the quantitative yield which is now actually attainable. Having achieved a yield of 93 per cent. of the easily hydrolyzable amide-N in the mercuric nitrate precipitate, as recorded in a parallel extract, it should be possible to obtain 84 per cent. of this fraction as crystalline glutamine.

The yields thus recorded, when taken in conjunction with the new analytical data presented, indicate that in all probability the whole of the easily hydrolyzable amide fraction of the dormant tubers is glutamine. There is thus a very strong presumption that when, during active metabolism, the easily hydrolyzable amide content of potato discs increases (**49**) the increase is due to glutamine. Thus, this more critical account of the analytical determination of this amide fraction, its conditions of hydrolysis, and behavior in the Van Slyke determination, together with its isolation, crystallization, and elementary analysis has vindicated the conclusion, drawn by STEWARD and PRESTON (**49**) from indirect evidence, that the easily hydrolyzable amide of actively metabolizing potato tissue was probably glutamine.

Evidence of the rôle of acid amides in metabolism

Despite much investigation the precise rôle of the acid amides in plants under diverse metabolic conditions is not certain, and the respective rôles of asparagine and glutamine is unknown. SCHULZE and PRIANISCHNIKOW (**45**) considered that in certain plants glutamine replaced asparagine as the chief amide and fulfilled the same function. VICKERY, PUCHER, and CLARK (**62**) and CLARK (**9**) have shown that tomato plants which contained both amides responded to ammonia supply by accumulating glutamine, but that the asparagine did not significantly increase in amount. GREENHILL and CHIBNALL (**21a**) and CURTIS (**12a**) have reported the exudation of glutamine from the leaves of grasses receiving nutrients rich in ammonia. MOTHES (**36**), on the other hand, using bean leaves, recorded asparagine rather than glutamine accumulation in response to ammonium salts. VICKERY, PUCHER, and CLARK (**62**) have suggested that in any green plant only one of the amides functions for ammonia detoxication and that the function of the second amide is by no means clear.

AMIDASES AS REGULATORS OF AMIDE CONTENT

It is tempting to consider that the marked contrast in their stability determines the respective rôles of these amides. Their formation from the

corresponding amino-acids in the plant appears to be governed by amidase or amidases. DERNBY (15) and LEVENE, SIMMS, and PFALTZ (33) concluded that the amides are hydrolyzed by an erepsin. Thus the amide group would be regarded as a peptide group in which one hydrogen atom of the amino group could be regarded as the equivalent of the amino acid residue of a dipeptide. This was endorsed by GROVER and CHIBNALL (22) who investigated the asparagine splitting power of extracts of barley roots and considered it unnecessary to postulate the existence of a specific asparaginase, regarding the action as due to a dipeptidase. GEDDES and HUNTER (19), however, obtained a preparation of yeast asparaginase which split the amide group quantitatively from asparagine, and had a slight activity on glutamine, but was quite inactive to all other acid amides tried. GRASSMANN and MAYR (20) were able to show that yeast asparaginase was an enzyme distinct from yeast dipeptidase and from aminopolypeptidase. There was evidence for a separate glutamine splitting enzyme in fresh autolyzed yeast. According to SCHWAB (48) yeast asparaginase is inactive to glutamine, but both an asparaginase and a glutaminase exist in barley. SCHWAB supposes that the proportion of the two amides in any given plant will be determined by the "active mass" of the enzymes, and he prefers to speak of asparaginase and glutaminase plants rather than of asparagine and glutamine plants.

CHIBNALL (7), using *Lolium perenne*, and VICKERY, PUCHER, and CLARK (62), using beet, have, more recently, shown the existence of active glutaminases in these plants. KREBS (30), working on animal tissues, has produced evidence for the existence of two distinct glutaminases ("brain type" and "liver type"), differentiated on the basis of their inhibition by glutamic acid and by different pH optima. Both enzymes are distinct from asparaginase. The balance of synthesis or decomposition of amide appeared to be influenced by pH and KREBS concluded that, in brain and retina, glutamine synthesis was not reversed since the hydrolysis only occurred in practice outside the physiological range of pH and that glutamine disappeared in these tissues without forming ammonia.

The rôle of amidase in the formation of amides by potato is strengthened by preliminary experiments here recorded which show that potato sap possesses amidase activity which passes to the protein-melanin precipitate formed on dialysis. The potato oxidase which deaminates amino acids also passes to the protein-melanin coagulum, but dialysis robs this preparation both of catechol compounds and of its ability to bring about deamination. In different samples of sap the relative amidase activity towards asparagine and glutamine is not constant, thus indicating that two distinct enzymes may be involved. It remains to be established, therefore, how far the metabolic behavior of the two amides may be interpreted in terms of the respective activity of their corresponding amidases.

RÔLE OF OXIDATION: ENERGY REQUIREMENTS IN AMIDE SYNTHESIS

The formation of either asparagine or glutamine from the ammonium

salts of the acid requires an energy supply. Thus the conversion of ammonium aspartate to asparagine at pH 7 involves a standard free energy of synthesis of 3460 cal. WILLARD (**68**) even considered that under conditions of adequate nitrogen nutrition the energy from the metabolites available for respiration limited asparagine synthesis in etiolated seedlings. KREBS (**30**) has shown that in kidney cortex and in brain tissue the synthesis of glutamine is linked with aerobic respiration and that hydrocyanic acid inhibits the synthesis of glutamine to approximately the same degree as it inhibits respiration. Under anaerobic conditions no glutamine synthesis takes place in these tissues.

It is only necessary here to recall that the biochemical survey (**49, 50, 51**) which prompted this investigation was instrumental in focusing attention upon the frequency with which the salt and oxygen effects upon the respiration of actively metabolizing potato cells found their parallel in effects upon nitrogen metabolism. The tissue first increases its content of glutamine (as we are now entitled to call the easily hydrolyzable amide) as it responds to oxygen supply by increased respiration, and the glutamine tends to reflect every subsequent change in the conditions which affect protein synthesis or respiration. CHIBNALL (**7**) also refers to observations that oxygen affects amide synthesis in plants. The oxygen which affects amide synthesis may be involved in oxidative deamination of amino-acids and/or in carbohydrate respiration. The necessity of carbohydrate for amide synthesis needs no emphasis, for it may act not only as a source of energy but also provide the carbon skeleton of the amide molecules.

A general concept of the factors which determine amide formation under a variety of metabolic or nutritional states should comprehend a range of circumstances which may extend from: (a) the primary synthesis of amides from an external source of nitrate or ammonia and in cells which, by virtue of their photosynthesis or storage products, have adequate carbohydrate available; to (b) the formation of amide from protein by degradation in senescent cells, which have been depleted of carbohydrate. The work on actively metabolizing potato cells falls into the former category; much classical work on excised leaves and etiolated seedlings as clearly falls into the second.

Under the conditions of protein breakdown it is possible to conceive that the energy for amide synthesis derives from the protein breakdown itself and that accumulation of ammonia in starved detached leaves marks the stage at which the necessary supply of N-free precursors (α-keto acids) for the carbon skeleton of the amides has been consumed. CHIBNALL (**7**) has shown that blades of perennial rye-grass infiltrated with the ammonium salt of α-ketoglutaric acid readily produce glutamine and that the whole of the metabolized ammonia is accounted for as nitrogen of new glutamine, together with a subsidiary amount of new asparagine. Furthermore, it was shown that the disappearance of ketonic acids showed good agreement with the amount of new amide formed and that the amount of α-ketoglutaric acid

which had disappeared was approximately equal to that demanded by the glutamine synthesized. VICKERY, PUCHER, and CLARK (62) showed that the increase in soluble nitrogen of beet roots treated with ammonium salts was accounted for by the increase in glutamine-N (i.e., by the sum of the amide and amino-N of glutamine) suggesting that the glutamine had been synthesized from N-free precursors.

In actively metabolizing potato cells adequate carbohydrate is available, and its metabolism releases energy far in excess of the requirements of all those concomitant processes (including protein synthesis) known to require energy. Under these conditions N-free precursors of amides may be presumed to arise as intermediate products of glycolysis or by oxidative deamination of stored amino-acids. STEWARD and PRESTON (49, 50, 51) used the tissue in such a way that, usually, it was dependent upon internal sources of nitrogen, and the ammonia for synthesis was shown to arise from amino-acids. However, external sources of inorganic nitrogen also equally well suffice as shown by experiments with nitrates (58) and ammonium salts (unpublished data).

The α-ketonic acids of potato tissue seem not to have been investigated nor have metabolic experiments involving infiltration of the tissues with either α-ketoglutaric or oxalacetic acid yet been made. STEWARD and PRESTON (49), however, have shown that the synthesis of protein, the utilization of amino-acids and asparagine, the formation of unstable amides, the utilization of organic acid radicals, and the oxidation phenomena involved in the browning reaction of living cells (which are in turn due to an enzyme system which produces oxidative deamination of amino acids) are all linked with the rate of aerobic respiration and hence with the machinery of salt absorption. A biochemical study of the organic acid metabolism of potato discs, under conditions of rapid salt uptake, such as that being undertaken by HOAGLAND and his co-workers (27) with barley roots, is necessary to establish how far utilization of specific organic acids may be correlated with synthesis and the utilization of the amides.

AMIDES DIRECTLY INCORPORATED IN PROTEIN

The main protein present in potato tubers and the only protein fraction to have been extensively studied is the globulin tuberin. The amino-acid analyses of tuberin are incomplete. KRIESEL and BELOZERSKY (29) record the presence of 1.05 to 1.02 per cent. amide-N in the ash-free dried samples examined. WINTON and WINTON (71) record the presence of 4.6 per cent. glutamic acid. Aspartic acid has, however, not been identified in the products of hydrolysis, although the amount of glutamic acid present, even if it is all present as glutamine, would only account for some 42 per cent. of the amide-N. The relative requirements for asparagine and glutamine for incorporation as such in the synthesized protein cannot, therefore, be assessed. Potato discs synthesize protein under conditions conducive to active metabolism and salt accumulation (49, 50, 51), but it should not be

presumed that the protein synthesized by these vitally active cells is necessarily identical with the globulin tuberin which is a storage product dissolved in the salts of the cell sap. The relationship between asparagine and glutamine and protein synthesis may be much less direct than their incorporation as such into the molecule of protein so synthesized. It seems rather as though glutamine should be regarded as an active molecule more readily capable than other substances of donating nitrogen to molecules about to be synthesized into protein. The following discussion shows that there is mounting evidence that this may be the case.

Glutamine as an amino-N donator

Braunstein and Kritzman (**3, 4**) have demonstrated the presence in muscle tissue of an enzyme system which catalyzes the reversible anaerobic deamination of 1(+) glutamic acid, with the formation of alanine from pyruvic acid thus: 1(+) glutamic acid + pyruvic acid $\rightleftharpoons$ α-ketoglutaric acid + 1(+) alanine. This enzyme they termed aminophorase. Similarly they showed that aspartic acid could also act as a primary amino donator. Also aminophorase will effect the deamination of monocarboxylic amino acids (e.g., alanine) in the presence of either -ketoglutaric or oxalacetic acids acting as amino acceptors. It appeared that almost any α-amino-acid could be deaminated to, or formed from, the corresponding α-keto acid by the aminophorase. Thus amino transfer (transamination or "umaminierung") requires that one member of the reacting pair must be a dicarboxylic keto or dicarboxylic amino-acid. Glutamic acid or α-ketoglutaric acid are particularly active in this respect (**4**). Kritzman (**31, 32**) considered that two enzymes are involved: a glutamic aminophorase and an aspartic aminophorase. *The transamination reaction is a mechanism whereby interconversion of amino-acids can take place, and it is of particular interest to our discussion inasmuch as it assigns to the dicarboxylic amino acids a key position in this important biochemical process.*

Cohen (**10, 11, 12**), however, considers that the transamination reaction is more restricted in its operation than is indicated by the evidence of Braunstein and Kritzman. The work of Virtanen and Lane (**67**), which ascribes to aspartic acid and to transamination a key position in the nitrogen fixation and metabolism of leguminous plants, has also been criticized by Wilson (**69**). Nevertheless, the work of Schoenheimer and co-workers (**44**) on animal tissues and the recent application of the mass isotope technique to the study of protein metabolism in plants (**24, 66**) supports the theory that the dicarboxylic acids play a central rôle in protein metabolism. It was found that deamination and amination take place continuously. Interchange of amino-N between the free amino-acids and the amino acids combined in the protein must continuously occur. Whenever amino-acids or ammonia artificially enriched in N 15 content are supplied to the organism the content of N 15 in the dicarboxylic acids (particularly glutamic acid) is much higher than in any of the other amino-acids in the tissue, with the

possible exception of the amino-acid in which the N 15 was supplied. If Cohen is correct in regarding the transamination reaction as limited in range, then these results suggest that yet another mechanism must be sought to account for the rapid amino group interchange which is continuously proceeding in the living cell, and in this mechanism glutamic acid must be particularly active. Transaminase systems have not been extensively studied in plants and an examination of potato tissue for aminophorase activity has yet to be undertaken.

Canalization of protein synthesis through glutamine

Earlier papers on the biochemistry of salt absorption in potato discs (**49, 50, 51**) have been concerned with the synthesis of protein in the tissue immersed in aerated distilled water and in aerated solutions of potassium and calcium salts. Protein synthesis was greater in potassium salt solutions than in water, and it could be suppressed by relatively strong calcium salt solutions. The nitrogen incorporated into the new protein was accounted for by the loss of nitrogen from the soluble nitrogen fraction. The soluble nitrogen was fractionated into α-amino-acid-N, heat-stable amide-N (asparagine amide-N), and heat-labile (easily hydrolyzable) amide-N; and the relative utilization of the different fractions as sources of nitrogen for protein synthesis was investigated. In the normal synthesis of protein the nitrogen utilized was mainly derived from the α-amino acids, and when the nitrogen was derived from this source there obtained the closest parallelism between protein synthesis and respiration. Under conditions of normal protein synthesis the heat-stable amide fraction only contributed a small fraction of the required nitrogen. Although amino-N was utilized in protein synthesis, *the concentration of amino-N did not appear to regulate the rate of synthesis.* The heat-stable asparagine amide-N decreased under the conditions which favored synthesis, but the easily hydrolyzable amide-N was found to increase. When, however, protein synthesis was accentuated, as in the strong potassium solutions, there occurred a marked decrease in the content of easily hydrolyzable amide without any similar effect upon the reserve of stable amides. The easily hydrolyzable amides were therefore tentatively regarded as reactive intermediates between the stable reserves of amino-acids and stable amide on the one hand and proteins on the other. The ammonia for formation of the unstable amides was regarded as having its origin in the oxidative deamination of amino acids, in the hydrolysis of asparagine or in the reduction of nitrate.

The identity of the easily hydrolyzable amide fraction of potato tissue with glutamine, in conjunction with the evidence already discussed, emphasizes the importance of glutamic acid as a donator of amino-N in protein synthesis. Transamination indicates how the amino-N of glutamic acid may be transferred to form other amino acids. As yet, however, we do not know how far glutamine itself accounts for all the glutamic acid which is metabolized since free glutamic acid may be a constituent of the

amino-N fraction which has not yet been fractionated. Whether the amide group of glutamine, which reacts like amino-N in the Van Slyke determination, may also be effective as an amino-N donator in the transamination reaction cannot yet be answered categorically. The only evidence on this point (**67a**) is inconclusive, but it does indicate greater reactivity of the amide-N of glutamine in this respect, than of asparagine. If the amide, as well as the amino, group of glutamine can enter into the transamination reaction and as, in view of its greater reactivity, the glutamine amide group is also the most labile source of ammonia for amino-acid formation from keto acids, then it becomes evident that this substance is an exceptionally versatile nitrogen donator to nitrogen free compounds. This may well be the key to the special significance of glutamine in protein synthesis and may account for the evident fact that, en route from soluble nitrogen reserves, the course of protein synthesis in the cells of potato tuber is canalized through glutamine.

The present paper has concentrated upon the amide fractions of potato tissue. Further work on the biochemistry of potato tissue under conditions conducive to active metabolism is still required. The need to investigate the amidase activity and the transamination system of potato in relation to its glutamine content arises directly from the present paper. Much, however, still requires to be learned of the identity of the amino-acids and organic acids of the potato tuber and of their relation to protein synthesis and respiration, in order that the biochemistry of potato tissue, under the conditions which are conducive to active metabolism and to the accumulation of salts from dilute solutions, can be adequately comprehended.

Summary of main conclusions

I

For purposes of extracting amides, aqueous extracts are preferable to 70 per cent. alcoholic extracts of fresh tissue. Aqueous extracts are not as selective as alcoholic ones and contain protein coagulable by heat. The amount of the amides extracted is not affected by drying the tissue before extraction. Extracts of dry tissue, however, contain less protein than extracts of fresh. Prior to the determination of the soluble nitrogen fractions protein is removed by heat coagulation. Though the quantity so removed is less than by use of various precipitants, including alcohol, this avoids contaminating the extracts with reagents which require to be removed before the analyses. Alcohol proves to be the most drastic of the protein-N precipitants with the exception of tungstic acid. The nitrogen of the aqueous extracts is not completely accounted for by heat coagulated protein, ammonia-N, easily hydrolyzable amide-N, asparagine amide-N, and true amino-N. It would be consistent with all these facts if the aqueous extracts contained other more complex, probably basic, substances which are absent from alcoholic extracts and disappear on purification. A complete account of the alcohol soluble nitrogen and of the nitrogen content of puri-

fied aqueous extracts can be given in terms of ammonia-N, easily hydrolyzable amide-N, asparagine amide-N, and true amino-N. Methods which give the best results for the determination of these fractions of the soluble nitrogen are described.

Ammonia-N should be determined by distillation under reduced pressure at 40° C.; distillation of ammonia in a micro-Kjeldahl apparatus causes some hydrolysis of amides. The determination of total amide-N presents no difficulty. In determining easily-hydrolyzable, amide-N, however, the hydrolysis of both pure glutamine and the easily-hydrolyzable amides of potato extracts was retarded by alcohol. Therefore, to avoid low results alcohol should be removed by distillation under reduced pressure before determining these amide fractions. It is verified that the true amino-N content of these extracts is obtained by determining amino-N (Van Slyke) and subtracting 80 per cent. of the easily hydrolyzable amide-N. The behavior of the easily hydrolyzable amide-N fraction under these analytical procedures is consistent with this substance being glutamine.

II

The effect of lead acetate precipitation followed by mercuric nitrate precipitation or precipitation by the Neuberg and Kerb reagent (mercuric acetate) on all the soluble nitrogen fractions was determined as well as the effect of phosphotungstic acid on the nitrogen compounds after liberation from the mercuric precipitates. In the light of these results the procedure for preparing crystalline amides from potato tuber was perfected. It depends upon precipitation of mercuric nitrate compounds from the filtrate after lead acetate precipitation and crystallization from the solutions of the nitrogen compounds prepared by decomposing the mercuric compounds. Crystallization was practiced first from aqueous, then from solutions progressively stronger in alcohol.

Two parallel batches of 2.5 and 3.5 kgm. of fresh tissue were worked up by the method prescribed. At each stage analyses of all the principle nitrogen fractions show the course of the purification; 71.5 per cent. of the easily hydrolyzable amide of the fresh tissue was recovered in the crystalline products. This represented 98 per cent. of this amide in the mercuric nitrate precipitates which in one series contained 93 per cent. of the easily hydrolyzable amide of the fresh tissue; hence it should have been possible, but for one inadvertent circumstance, to obtain crystalline deposits containing 91 per cent. of the easily hydrolyzable amide of the tissue and to have recovered 80 per cent. of the easily hydrolyzable amide-N of the initial extract as crystalline glutamine.

III

Crystalline products containing the amides were submitted to repeated crystallizations. By a process described, tyrosine, glutamine, and asparagine were prepared in pure form. The yields obtained corresponded to

0.116 gm. tyrosine; 1.42 gm. asparagine; and 1.51 gm. glutamine per 1000 gm. of fresh tuber tissue. This accounts for 66 per cent. of the easily hydrolyzable amide-N contained in the fresh tissue as crystalline glutamine; for 61 per cent. of the stable amide-N of the tissue as crystalline asparagine; and 36 per cent. of the Van Slyke amino-N of aqueous extracts as amide-N and as tyrosine. Elementary analyses, melting points, and analyses of amide- and amino-N content of the crystalline products are all consistent with their identity as the pure substances designated. The yield of glutamine obtained is an advance upon any previous report and the method described should make it possible to isolate glutamine in high yield even when asparagine is present in greater quantity.

IV

The identification of the easily hydrolyzable amide fraction of potato as glutamine together with the known reactions of this substance, re-emphasizes the key position already assigned to this amide in protein synthesis.

That synthesis and hydrolysis of the amides may proceed under specific amidases is noted and also that amidase activity may well be influenced by pH (by analogy with enzyme preparations from animal organisms) after dialysis. The amidase activity of potato sap can be distinguished from potato oxidase and its oxidative deamination. In leaves, the quantitative synthesis of glutamine from α-ketoglutaric acid has been established, but there is little direct evidence for potato tissue beyond the fact that organic acid radicles are consumed during synthesis.

The bearing of glutamine on protein synthesis does not derive from its incorporation, as such, in the protein but is due much more to its ability to donate nitrogen to other molecules about to be synthesized into protein. "Transamination," whereby amino groups are transferred enzymatically from amino-acids of dicarboxylic acids to a keto acid, is important because glutamic acid is particularly active in this respect and may be reconstituted from other amino-acids (e.g., of monocarboxylic acids) which do not otherwise enter into the transamination reaction. It is still inconclusive whether the amide group of glutamine behaves like an amino group under the influence of the enzyme which procures transamination. Be this as it may, the reactive amide group of glutamine is the most labile source of ammonia for synthesis. Therefore, the significance of glutamine seems to be that as the soluble nitrogen passes through this substance the amino-groups become particularly susceptible of being "donated" during amino-acid formation, and the amide group constitutes a particularly labile source of nitrogen for synthesis.

Further investigation of the amidase and the supposed aminophorase systems of potato are necessary, and the nutritional effects of α-keto acid supply should also be determined. The biochemical survey of actively metabolizing potato discs should now be extended to identify the other components of the amino-N fraction and possibly also the alcohol insoluble

nitrogen compounds. This is necessary to specify the range of substances from which nitrogen is withdrawn and canalized through glutamine en route to the more complex substances.

The work at Birkbeck College was interrupted by war conditions. The junior author was able to complete the experimental work at the University of Manchester where he enjoyed facilities for which thanks are now expressed.

The comprehensive paper by ARCHIBOLD on the chemistry of glutamine and its rôle in animals and plants appeared after this paper was prepared; it will be found in Chemical Reviews **37**: 161–208. 1945.

BOTANY DEPARTMENT
BIRKBECK COLLEGE
UNIVERSITY OF LONDON

LITERATURE CITED

1. AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS. Reports of Committee on Methods of Analysis. The determination of nitrogen in relative simple compounds. Plant Physiol. **2**: 205–211. 1927; **10**: 393. 1935.
2. BLISH, M. J. A study of the non-protein nitrogen of wheat flour. Jour. Biol. Chem. **33**: 551–559. 1918.
3. BRAUNSTEIN, A. E., and KRITZMAN, M. G. Formation and breakdown of amino acids by intermolecular transfer of the amino group. Nature **140**: 503–504. 1937.
4. ————————, and ————————. Catalytic function of amino and keto dicarboxylic acids in amino nitrogen transfer between monocarboxylic substrates. X. Formation and decomposition of amino acids by intermolecular transfer of amino groups. Biochimia **4**: 303–315. 1939.
5. CAMPBELL, W. R., and HANNA, M. I. The determination of nitrogen by modified Kjeldahl methods. Jour. Biol. Chem. **119**: 1–7. 1937.
6. CHIBNALL, A. C. A new method for the separate extraction of vacuole and protoplasmic material from leaf cells. Jour. Biol. Chem. **55**: 333–342. 1932.
7. ————————. Protein metabolism in the plant. New Haven. 1939.
8. ————————, and WESTALL, R. G. The estimation of glutamine in presence of asparagine. Biochem. Jour. **26**: 122–132. 1932.
9. CLARK, H. E. Effect of ammonium and of nitrate nitrogen on the composition of the tomato plant. Plant Physiol. **11**: 5–24. 1936.
10. COHEN, P. P. Transamination with purified enzyme preparations (transaminase). Jour. Biol. Chem. **136**: 565–584. 1940.
11. ————————. Kinetics of transaminase activity. Jour. Biol. Chem. **136**: 585–601. 1940.
12. ————————, and HEKHIUS, G. L. Rate of transamination in normal tissues. Jour. Biol. Chem. **140**: 711–724. 1941.

12a. CURTIS, L. C. Exudation of glutamine from lawn grass. Plant Physiol. **19**: 1–5. 1944.

13. DAMODARAN, M. The isolation of asparagine from an enzymic digest of edestin. Biochem. Jour. **26**: 235–247. 1932.

14. ————————, JAABACK, G., and CHIBNALL, A. C. The isolation of glutamine from an enzymic digest of gliadin. Biochem. Jour. **26**: 1704–1713. 1932.

15. DERNBY, K. G. Studien über die proteolytischen Enzyme der Hefe und ihre Beziehung zu der Autolyse. Biochem. Zt. **81**: 107–208. 1917.

16. EISENSCHIMMEL, W. Studie über Glutamin. Z. Zuckerund. Cechslov. Rep. **51**: 337–347. 1927.

17. FOLIN, O., and WU, H. A system of blood analysis. Jour. Biol. Chem. **38**: 81–110. 1910.

18. FOREMAN, F. W. The transformation of glutamic acid into 1-pyrrolidone carboxylic acid in aqueous solution. Biochem. Jour. **8**: 481–493. 1914.

19. GEDDES, W. F., and HUNTER, A. Observations on the enzyme asparaginase. Jour. Biol. Chem. **77**: 197–229. 1928.

20. GRASSMANN, W., and MAYR, O. Zur Kenntnis der Heferasaparaginase. Hoppe-Seyl. Zt. **214**: 185–210. 1933.

21. GREENWALD, I. The estimation of non-protein nitrogen in blood. Jour. Biol. Chem. **21**: 61–68. 1915.

21a. GREENHILL, A. W., and CHIBNALL, A. C. Exudation of glutamine from perennial rye-grass. Biochem. Jour. **28**: 1427. 1934.

22. GROVER, C. E., and ————————. The enzymic deamination of asparagine in the higher plants. Biochem. Jour. **21**: 857–868. 1927.

23. HART, E. B., and BENTLEY, W. H. The character of the water-soluble nitrogen of some common feeding stuffs. Jour. Biol. Chem. **22**: 477–483. 1915.

24. HEVESY, G., LANGSTROM-LANG, K., KESTON, A. S., and CARSTEN, O. Compt. rend. trav. lab. Carlsberg. Ser. Chim. **23**: 213. 1940.

25. HILLER, A., and VAN SLYKE, D. D. A study of certain protein precipitants. Jour. Biol. Chem. **53**: 267. 1922.

26. HIRSCH and LUDWIG. Cited by HUSEMANN, A., and KRAUT, K. Supplement Band zu Gmelin's Handbuch der Chemie. p. 898. 1868.

27. HOAGLAND, R. D. Lectures on the inorganic nutrition of plants. Chronica Botanica, Waltham, Massachusetts. 1944.

28. HUNGERBUHLER, J. Composition of unripe tomatoes. Landw. Vers, Sta. **32**: 381–388. 1885.

29. KREISEL, A., and BELOZERSKY, A. DIE. Eiweisstoffe der Blätter und der Knollen der Kartoffel. Hoppe-Seyl. Zt. **226**: 77–79. 1934.

30. KREBS, H. A. Metabolism of amino-acids. IV. The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. Biochem. Jour. **29**: 1951–1969. 1935.

31. KRITZMAN, M. G. Das Ferment der Glutaminssäure. Umamierung V. Bildung und Zerfall von Aminosäuren durch intermolekulare Umlagerung von Aminogruppen. Biochimia **3**: 603–615. 1928.
32. ————. The enzyme system transferring the amino group of aspartic acid. Nature **143**: 603–604. 1939.
33. LEVENE, P. A., SIMMS, H. S., and PFALTZ, M. A. The relation of chemical structure to the rate of hydrolysis of peptides, III. Enzyme hydrolysis of depeptides and tripeptides. Jour. Biol. Chem. **70**: 253–264. 1926.
34. MELVILLE, J. Labile glutamine peptides and their bearing on the origin of the ammonia set free during the enzymic digestion of proteins. Biochem. Jour. **29**: 179–186. 1935.
35. MERRILL, A. T. A note on the relation of pH to tungstic acid precipitation of protein. Jour. Biol. Chem. **60**: 257–259. 1924.
36. MOTHES, K. Ein Beitrag zur Kenntnis des N-Stoffwechsels höherer Pflanzen. Planta **1**: 472–552. 1926.
37. NEUBERG, C., and KERB, J. Über ein Fällungsmittel für Aminosäuren. Biochem. Zt. **40**: 498–512. 1912.
38. NEUBERGER, A., and SAUGER, F. The nitrogen of the potato. Biochem. Jour. **36**: 662–671. 1942.
39. PLIMMER, R. H. A. The action of nitrous acid upon amides and other "amino" compounds. Jour. Chem. Soc. (London) **127**: 2651–2659. 1925.
40. PRAGER, BERNHARD, and JACOBSON, PAUL. Beilstein's Handbuch der organischen Chemie. Band IV. 1922.
41. PUCHER, G. W., LEAVENWORTH, C. S., and VICKERY, H. B. Determination of total nitrogen of plant extracts in presence of nitrates. Ind. & Eng. Chem. Anal. Ed. **2**: 191–193. 1930.
42. ————, and VICKERY, H. B. Determination of glutamine in plant tissues. Ind. & Eng. Chem. Anal. Ed. **12**: 27–29. 1940.
43. RUMSEY, L. A. The precipitation of proteins from cereal extracts by sodium tungstate. Ind. & Eng. Chem. **15**: 270–272. 1923.
44. SCHOENHEIMER, R. The dynamic state of body constituents. Harvard Press. 1942.
45. SCHULTZ, E. Abscheidung des Asparagine aus Flüssigkeiten. Ber. d. chem. Ges. **15**: 2855–2856. 1882.
46. ————, and BOSSHARD, E. Über das Glutamin. Ber. d. chem. Ges. **16**: 312–315. 1883.
47. ————, and ————. Allantoin, asparagine, hypoxanthin and guanin as plant constituents. Hoppe-Seyl. Zt. **9**: 420–444. 1885.
48. SCHWAB, G. Studien über Verbreitung und Bildung der Säureamide in der höheren Pflanzen. Planta **25**: 579–606. 1936.
48a. STEWARD, F. C. Experimental conditions which determine salt absorption by storage tissue. Protoplasma **15**: 29–58. 1932.

49. ————, and PRESTON, C. Metabolic processes of potato discs under conditions conducive to salt accumulation. Plant Physiol. **15**: 23–61. 1940.

50. ————, and ————. The effect of salt concentration upon metabolism of potato disc and the contrasted effect of potassium and calcium salts which have a common ion. Plant Physiol. **16**: 86–116. 1941.

50a. ————, and ————. Effects of pH and the components of bicarbonate and phosphate buffered solutions on the metabolism of potato discs and their ability to absorb ions. Plant Physiol. **16**: 481–519. 1941.

51. ————, STOUT, P. R., and PRESTON, C. The balance sheet of metabolites for potato discs showing the effect of salts and dissolved oxygen on metabolism at 23° C. Plant Physiol. **15**: 409–447. 1940.

52. THOMAS, W. Nitrogenous metabolism of *Pyrus malus* L. I. Influence of temperature of desiccation on water-soluble nitrogenous constituents and separation of water-soluble protein from non-protein constituents. Plant Physiol. **2**: 550–566. 1927.

53. VAN SLYKE, D. D. The gasometric determination of aliphatic amino nitrogen in minute quantities. Jour. Biol. Chem. **16**: 121–124. 1913.

54. ————. Note on the micro-method for gasometric determination of aliphatic amino nitrogen. Jour. Biol. Chem. **23**: 407–409. 1915.

55. VAUQUELIN, Cited by GMELIN, L. Handbook of chemistry. English translation by H. WATTS. **10**: 239. 1856.

56. VICKERY, H. B. Some nitrogenous constituents of the juice of the alfalfa plant. I. The amide and amino acid nitrogen. Jour. Biol. Chem. **60**: 647–655. 1924.

57. ————. Some nitrogenous constituents of the juice of the alfalfa plant. II. The basic nitrogen. Jour. Biol. Chem. **61**: 117–127. 1924.

58. ————. The nitrogenous constituents of green plants. Ann. Rev. Biochem. **1**: 611–617. 1932.

59. ————. Glutamine and asparagine in tobacco leaves. Jour. Biol. Chem. **113**: 157–160. 1936.

60. ————, and PUCHER, G. W. A stillhead for rapid concentration in vacuo. Ind. & Eng. Chem. Anal. Ed. **6**: 373. 1934.

61. ————, ————, and CLARK, H. E. The preparation of glutamine. Jour. Biol. Chem. **109**: 39–42. 1935.

62. ————, ————, and ————. Glutamine metabolism of the beet. Plant Physiol. **11**: 413–422. 1936.

63. ————, ————, ————, and CHIBNALL, A. C., and WESTALL, R. G. The determination of glutamine in the presence of asparagine. Biochem. Jour. **29**: 2710–2712. 1935.

64. ————, ————, and LEAVENWORTH, C. S. Determination of ammonia and of amide nitrogen in plant tissue. End. & Eng. Chem. Anal. Ed. **7**: 152–156. 1935.

65. ————, ————, ————, and WAKEMAN, A. J. Chemical researches on the tobacco plant. V. Chemical changes occurring during growth. Connecticut Agr. Exp. Sta. Bull. 374. 1935.

66. ————, ————, SCHOENHEIMER, R., and RITTENBERG, D. The assimilation of ammonia nitrogen by the tobacco plant; a preliminary study with isotopic nitrogen. Jour. Biol. Chem. **135**: 531–539. 1940.

67. VIRTANEN, A. I., and LAINE, T. Biological synthesis of amino-acids from atmospheric nitrogen. Nature **141**: 748–749. 1938.

67a. ————, and ————. Über die Umamierung in grünen Pflanzen. Biochem. Zt. **308**: 213–215. 1941.

68. WILLARD, L. MCRAY. Studies on the biochemical synthesis of asparagine. Arch Biochem. **1**: 197–205. 1942.

69. WILSON, P. W. The biochemistry of symbiotic nitrogen fixation. University of Wisconsin Press, Madison. 1940.

70. WINTERSTEIN, E. Isolierung von Aminosäuren, Asparagin, Glutamin usw aus Pflanzen. Abderhalden Handbuch d. biochem. Arbeitsmethoden. Berlin. 1923.

71. WINTON, A. I., and WINTON, K. B. The structure and composition of foods. Vol. II. John Wiley & Sons. New York. 1935.

72. WOOLF, B. The estimation of ammonia, urea, and total nitrogen. Chem. Ind. Rev. **6**: 1060. 1928.

THE EFFECT OF VARIOUS IONS ON GUTTATION OF THE TOMATO[1]

G. J. Raleigh

Introduction

In a previous publication (6) attention was called to the fact that in an experiment with tomato plants, those supplied with complete nutrient solution guttated while those in solutions lacking both nitrate and ammonium nitrogen did not guttate. At that time it was thought that the low pH of the solutions might have been responsible for the lack of guttation.

Two additional observations made during the course of later work have suggested that pH alone may not have been responsible. On May 8, 1942, all plants in complete nutrient solutions that had been changed three days previously were guttating markedly while others in solutions that had not been changed were not guttating. In neither instance was the pH low. On May 19, 1942, plants that had been allowed to grow in complete solutions for 18 days but were supplied with additional calcium during the interval were treated as follows by adding concentrated solutions to the depleted solutions:

1. Complete solution (Hoagland solution #2).
2. Complete solution but all nitrogen from urea.
3. Solution lacking nitrogen.
4. Solution lacking phosphorus.
5. Solution lacking potassium.
6. Solution lacking calcium.
7. Check (water only added).

On May 20, a very humid day, guttation water was dropping from all the plants in treatments 1 and 6, and none was visible on any plant in any other treatment. The pH of the urea and of the minus-nitrogen solutions was low (pH 4.6), but that of all of the other solutions was about pH 6 except that of the check solution which was nearly neutral. These observations suggested the possibility that addition of an element to a nutrient solution low in that element might cause guttation of plants that had been checked in growth by the deficiency of the element.

Grossenbacher (2) in the course of his work on diurnal fluctuation in root pressure noted that plants transferred to fresh, dilute, but balanced nutrient solutions respired appreciably more and developed much more root pressure than those transferred to dilute $CaSO_4$. Hamner (3) found that nitrate increased the respiration of tomato and of wheat plants with high carbohydrate reserves. Hoagland and Broyer (5) suggested that their finding concerning the effect of carbohydrate reserves on salt absorp-

[1] Paper no. 276. Department of Vegetable Crops, Cornell University, Ithaca, New York.

tion had implications as far as root pressure was concerned. In later work, BROYER and HOAGLAND (1) showed the importance of the relation of root metabolism to the upward movement of salt. Guttation was greater on plants which were originally grown with the smaller supplies of salts and were initially higher in sugars.

Procedure

With the exception of the experiment with potted plants grown in soil, the strain of tomato used in these experiments was one selected from a single plant in a uniform field of Bonny Best. Seed was grown for two generations on isolated plants. Single plants were grown in crocks of 6-liter capacity in Hoagland's #2 solution with A5 minor elements added[2] until they had reached a height of approximately 130 cm. before they were changed to solutions lacking a single nutrient. They were then allowed to grow in the deficient solution until deficiency symptoms were evident. Care was taken to maintain the supply of all nutrients except the deficient one. This was done by changing the solutions or by the addition of equivalent chemicals. The pH of the solutions lacking nitrogen was adjusted every other day with NaOH. A lack of a test for nitrates in any part of the plant as indicated by diphenylamine was considered evidence of nitrate deficiency. At this stage the iodine test for starch indicated high carbohydrate reserves, as would be expected. The diphenylamine test was not relied on in the single experiment with Hubbard squash in culture solution. In that experiment, yellow color of the lower leaves and markedly retarded growth were used as an index of nitrogen deficiency even though the petioles of the older yellow leaves gave a test for nitrate with diphenylamine.

When deficiency symptoms were noted, the plants were transferred from the greenhouse to an underground room with porous brick walls which had been thoroughly soaked with water prior to the experiment and which were kept wet during the course of the work. Solutions were added, and the time required for guttation was noted. In preliminary experiments it made no difference if a complete solution or a solution of a compound containing the deficient element was added to the deficient solutions. In the work reported here the latter procedure was followed. The relative humidity of the room ranged from 93 to 96 per cent. and the temperature varied within 2 degrees during any one experiment and from 68° to 74° F. for all experiments.

In these experiments it was difficult to find a satisfactory index for amount of guttation. Even though much of the solution guttated by the plants was lost in collecting it by placing a test tube under each drop, that method was considered as satisfactory as any. In all of the following experiments where guttation is indicated as marked, it was possible to collect from 1 to 3 ml. of solution from the plants at one time and to repeat the collection after a few hours. In no experiment, except that mentioned under "THE EFFECT OF NITRATE AND OF AMMONIUM NITROGEN ON GUTTATION" did

[2] The solutions used in these experiments were essentially those given in citation (4).

a plant guttate if grown in a solution deficient in an element and not supplied with that element when the plants were placed in the moist room.

Experiments with nitrogen

The effect of nitrate and of ammonium nitrogen on guttation.—Plants grown from seed sown April 9, 1942, were kept in complete solutions until July 25 at which time the solutions were low in nitrogen as indicated by the diphenylamine test. On July 25 they were transferred to dilute solutions lacking nitrogen and on July 29 were placed in the moist room. Solutions of $NaNO_3$ and of NH_4Cl were added to the deficient solutions to make the concentration of $NaNO_3$ and of NH_4Cl 0.006 M in respective treatments. To another lot of 4 plants water was added. As indicated in table I the plants with nitrate added began to guttate after 6 hours. The plants with ammonia added did not guttate as rapidly, but all were guttating in 19 hours. One of the plants allowed to remain deficient in nitrogen showed a very small amount of guttation at 2:30 P.M. but not a sufficient quantity to make it possible to collect any of it, and no more was evident after that

TABLE I

Time required for guttation to become apparent and amount of solution collected from plants following the addition of $NaNO_3$ and of NH_4Cl to nitrogen-deficient solutions. Experiment started 11:00 A.M.

Treatment		5:00 P.M.	2:30 A.M.	6:30 A.M.	8:00 A.M.	4:00 P.M.
$NaNO_3$—0.006 M		All	.....	.....	.....	.....
NH_4Cl—0.006 M	1	.....	X	.....	.....	3.1 ml.
	2	.....	.....	X	.....	1.2 "
	3	.....	.....	X	.....	0.5 "
	4	.....	.....	.....	X	0.1 "

time. None of the other three plants in solutions to which water was added guttated. During the course of the experiment the solutions supplied with NH_4Cl were tested for pH three times and NaOH added to raise the pH to approximately 6.5. The amount of guttation water collected from the plants with NH_4Cl at the 19th hour is shown in table I. The nitrate plants guttated profusely.

Microchemical tests indicated the presence of nitrates and of ammonia, respectively, in the guttated solutions soon after it was possible to collect solutions from plants supplied with nitrates and those in solutions to which ammonium chloride had been added.

The experiment was repeated in May, 1944. The plants were moved to the moist room at 10:00 A.M. At 10:00 P.M. two plants supplied with nitrate were guttating. The plants were not seen again until 8:00 A.M. the following day at which time the other two plants with nitrate were guttating. The plants supplied with NH_4Cl were guttating at 11:00 A.M. and 2:30 P.M. of the second day. The plants in solutions to which water alone was added did not guttate.

On the third day the plants were tested for nitrates, using the xylenol method. The leaflets of the check, the ammonia and the nitrate plants

tested, respectively, 10, 20 and 150 p.p.m. of nitrate nitrogen. Guttated solution collected at that time from the nitrate plants contained 12 p.p.m. The difference in the nitrate content of the check plants as compared with those supplied with NH_4Cl cannot be considered significant due to the inadequate number of tests made.

EXUDATION FROM DECAPITATED PLANTS SUPPLIED WITH NITRATES.—In this experiment plants grown from seed sown on May 28, 1942, were kept in complete solutions until July 27 at which time they were transferred to solutions lacking nitrogen. On August 21 nine plants were transferred to the moist room, and the tops of three of the plants were cut off six inches above the root. The other six plants were not decapitated until six hours later when guttation was becoming evident on the three of them in solutions to which $NaNO_3$ had been added to supply 0.006 M $NaNO_3$. The total

TABLE II

AMOUNT OF BLEEDING FROM DECAPITATED PLANTS IN NITROGEN-DEFICIENT SOLUTIONS SUPPLIED WITH NITRATE (IN ML.). EXPERIMENT STARTED AUGUST 21 AT 11:00 A.M.

TREATMENT		AUG. 22 8:00 A.M.	AUG. 23 9:00 A.M.	AUG. 24 8:00 A.M.	AUG. 25 8:00 A.M.	AUG. 26 9:00 A.M.	AUG. 28 8:00 A.M.
Nothing added	1	20	45	81	100	130	154
decapitated 5:00 P.M.	2	11	36	64	98	108	116
	3	20	58	84	110	130	161
$NaNO_3$—0.006 M	1	70	150	207	230	270	323
decapitated 5:00 P.M.	2	176	392	494	550	600	650
	3	170	360	555	680	890	1215
$NaNO_3$—0.006 M	1	58	154	223	275	335	435
decapitated 11:00 A.M.	2	265	427	540	640	720	860
	3	163	318	408	532	642	767

amounts of bleeding for each of the six days following the beginning of the experiment are shown in table II. There was considerable variation in the amount of bleeding within the two treatments receiving nitrate but no significant differences between those that had been decapitated at the beginning of the experiment and those cut off after they began to guttate. No plants with nitrates bled less than twice as much as the maximum amount from a plant in nitrogen-deficient solution. The average of those receiving nitrates was approximately five times greater than those in deficient solutions.

EFFECT OF NITRATE SOLUTIONS ON THE GUTTATION OF POTTED PLANTS.—Tomato plants of the Earliana variety seeded February 10, 1943, and set into potting soil on February 27 were selected on the basis of foliage appearance and a lack of nitrate in the foliage (diphenylamine test) and placed in the moist room. At that stage the plants were 27 cm. tall. Eight plants were given 200 ml. of $NaNO_3$ (0.012 M) and a similar number the same quantity of distilled water. All of the plants supplied with nitrate were guttating after four hours, and guttation was marked after six hours;

none of those given distilled water guttated during the two days of the experiment.

EFFECT OF NITRATE SOLUTIONS ON GUTTATION OF SQUASH PLANTS.—On July 28, 1944, eight Hubbard squash plants were transferred to the moist room. They were seeded June 20 and grown in nitrogen-deficient solutions from July 20 after having been in complete solutions for twenty days. Sufficient $NaNO_3$ was added to the nitrogen-deficient solutions to make the $NaNO_3$ content 0.005 M. No nutrients were added to the solutions of the other four plants. Five hours later all of the plants in the nitrate solutions were guttating. The plants in nitrogen-deficient solutions did not guttate during a 2-day period.

EXPERIMENTS WITH PHOSPHORUS

On May 4, 1944, eight plants seeded December 20, 1943, were placed in the moist room. They had been grown since March 14 in phosphorus-deficient solutions and were showing phosphorus-deficiency symptoms as evidenced by bronzing of the leaves and reduced growth. Enough KH_2PO was added to the phosphorus-deficient solutions of one-half of the plants to make the concentration of KH_2PO_4 0.001 M, and the remainder were used as checks. After seven hours one plant given phosphorus was guttating; two more were guttating after twenty hours (not seen in interval); and the fourth after twenty-three and one-half hours. Marked guttation continued after that time. None of the plants in solutions lacking phosphorus guttated.

EXPERIMENTS WITH POTASSIUM

Twelve plants seeded April 9, 1942, and grown in complete solutions from May 5 to July 11, were transferred to potassium-deficient solutions on that date. On August 5 they were placed in the moist room. To the solution of 4 of the plants enough KCl was added to make the concentration of KCl 0.006 M, four were supplied with KNO_3 (0.006 M) and to four, no chemicals were added. None of the plants without potassium guttated. The degree of guttation following the adding of the two salts of potassium is shown in table III.

The experiment was repeated in May, 1944, with similar results except that two of five plants supplied with KCl did not guttate. When these two plants were sectioned longitudinally after the experiment, many brown areas were found in the stems. This necrosis may have been due to excessive potassium deficiency.

EXPERIMENTS WITH CALCIUM AND MAGNESIUM

Two experiments with calcium made in 1942 and 1943 and one with magnesium made in 1944 gave negative results. Plants showing marked calcium deficiency or marked magnesium deficiency did not guttate when the respective nutrients were added to the deficient solutions.

Discussion

The addition of nitrates to nitrogen-deficient solutions caused marked guttation of plants that had grown in such solutions until no nitrate nitrogen was present in any of the tissues as indicated by the diphenylamine test. At that stage, the plants were high in starch as indicated by the iodine test. Presumably, the supplying of nitrate to such plants caused an increase in respiration as shown by HAMNER (3). Ammonium nitrogen, when substituted for nitrate, produced similar results, but the time required for guttation to start was longer. It is difficult to evaluate the part played by the oxygen from the nitrate. The fact that plants in solutions containing nitrates guttated profusely in a shorter time than those in solutions with

TABLE III

TIME REQUIRED FOR GUTTATION TO BECOME APPARENT ON PLANTS FOLLOWING THE ADDITION OF POTASSIUM SALTS TO SOLUTIONS DEFICIENT IN POTASSIUM. EXPERIMENT STARTED 10: 00 A.M.

TREATMENT		5: 30 P.M.	9: 15 P.M.	MIDNIGHT	8: 00 A.M.
None added	1	.....	.....	.....	.....
	2	.....	.....	.....	.....
	3	.....	.....	.....	.....
	4	.....	.....	.....	.....
KCl—0.006 M	1	.....	Very slight	Considerable	Marked
	2	.....	Considerable	½ to marked	Marked
	3	.....	Slight	Slight	Considerable
	4	.....	Considerable	Marked	Marked
KNO_3—0.006 M	1	.....	Considerable	Marked	Marked
	2	Slight	Marked	Marked	Marked
	3	.....	Considerable	½ to marked	Marked
	4	Slight	Marked	Marked	Marked

ammonium nitrogen may have been the result of oxygen supplied by the nitrate. It may be that those given ammonia were delayed by the acidity resulting from its use even though the solutions were made neutral at frequent intervals. Quantities of nitrate and of ammonium nitrogen could be found by microchemical tests in guttated solutions soon after it was possible to collect such solutions. Because transpiration was markedly reduced by the saturated atmosphere in which these experiments were made, it is presumed that the nutrients in the guttated solutions could not have been carried in the "transpiration stream" but were the result of root activity.

The results of the second experiment with K indicate that injuries to some of the plants probably caused by the deficiency of K may have been responsible for a lack of guttation when solutions containing K were added to depleted solutions. Injury may also have been responsible for similar results in the experiments with Ca and Mg, and it is possible that guttation might have occurred had the plants been removed to the experimental room at an earlier stage in the development of deficiency of each of those elements.

No attempt was made to determine the mechanism responsible for guttation. If some specific mechanism is involved, it would seem that it is

activated by any one of a number of elements if its inactivation has been brought about by a lack of that element.

Summary

Tomato plants were grown in complete solutions and then transferred to solutions lacking nitrogen, phosphorus, potassium, calcium and magnesium, omitted singly until deficiency symptoms were clearly evident. When the lacking nutrient was supplied to the deficient solutions and the plants were kept in a room with relative humidity of about 94 per cent., marked guttation occurred in the plants supplied with nitrogen, phosphorus, and potassium respectively. Guttation did not occur on the plants in the deficient solutions or on those in solutions supplied with calcium or magnesium.

DEPARTMENT OF VEGETABLE CROPS
CORNELL UNIVERSITY
ITHACA, NEW YORK

LITERATURE CITED

1. BROYER, T. C., and HOAGLAND, D. R. Metabolic activities of roots and their bearing on the relation of upward movement of salts and water in plants. Amer. Jour. Bot. **30**: 261–273. 1943.
2. GROSSENBACHER, KARL A. Diurnal fluctuation in root pressure. Plant Physiol. **13**: 669–676. 1938.
3. HAMNER, KARL C. Effects of nitrogen supply on rates of photosynthesis and respiration in plants. Bot. Gaz. **97**: 744–764. 1936.
4. HOAGLAND, D. R., and ARNON, D. I. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Cir. 347. 1938.
5. ————————, and BROYER, T. C. General nature of the process of salt accumulation by roots with description of experimental methods. Plant Physiol. **11**: 471–507. 1936.
6. RALEIGH, G. J. Fruit abnormalities of tomatoes grown in various culture solutions. Proc. Amer. Soc. Hort. Sci. **37**: 895–900. 1939.

LENGTH OF LIFE OF ROOTS OF TEN SPECIES OF PERENNIAL RANGE AND PASTURE GRASSES[1, 2]

J. E. Weaver and Ellen Zink

(with six figures)

Introduction

It is well known that death of the tops of practically all prairie grasses occurs each fall in temperate grasslands where the soil is regularly frozen. Year after year new shoots replace the old ones in this vegetation of long-lived perennials. But as to what portion of the root system is retained and over what period of time, we are almost without information. This maintains despite the fact that much work has been done to increase our knowledge of the root systems of prairie grasses. Since the early studies of Weaver (6, 7) on their depth and lateral extent, detailed investigations have been made by Pavlychenko (1) upon their rate of growth, total root length, and quantity and quality of root material. The quantity of root material under different grassland climates has been ascertained by Shively and Weaver (2), and the quantity under different degrees of utilization of these grasses in the same climate by Weaver and Harmon (8). Weaver, Hougen, and Weldon (9) studied the amount of root material at different soil depths; but the length of life of the roots of prairie grasses, except for brief study by Stoddart (4), seems to have been entirely neglected. However, an investigation on the longevity of the seminal roots of certain grasses has recently been made by Weaver and Zink (10).

This lack of information is general for other grasses as well. Notable exceptions are the studies of Sprague (3) on Kentucky bluegrass and Colonial bent, and of Stuckey (5) on twelve species of cultivated grasses. Sprague concluded that at least one-half of the root system is newly generated each spring; Stuckey states that for some species the whole root system is regenerated annually.

Methods

Containers

Plants were grown from seed in large containers. Nine cylindrical cans made of heavy galvanized iron, 24 inches tall and 18 inches in diameter, were filled with loam potting soil. This had been screened, brought to an approximately uniform water content favorable for rapid growth, and thoroughly mixed. The soil had a hygroscopic coefficient of about 10 per cent. Openings in the bottom of the cans, covered with woven wire and an inch

[1] Contribution from the Department of Botany, University of Nebraska, no. 148.

[2] This investigation was aided by a grant to the senior author from The Graduate Council of The University of Nebraska, and grants to the junior author from the Nebraska Academy of Sciences.

depth of gravel, afforded adequate drainage. In filling the containers to near the top, the soil was tamped firmly in place. A removable extension consisting of a heavy galvanized iron band, 6 inches wide, riveted and soldered, and just large enough to fit within the container, was inserted to a depth of 2 inches. Here it rested on a number of rivet-heads just inside the metal rim. This removable extension, which was filled with soil consisting of well-mixed half sand and half loam, increased the depth of the container 4 inches. In addition, 26 heavy galvanized iron containers, 9 by 9 inches in diameter and 29.5 inches deep, were used. Each was fitted with a removable extension, which increased the depth to 33.5 inches, and permitted by its removal and the washing away of the sand-loam mixture easy access to the roots.

Seeds were planted early in March. Those of two species of grasses were planted on opposite sides of each of the large containers and about 4 inches from the side of the extension. Thus, seed of each of 9 species was planted in duplicate but in different large containers. A third planting of each was made in one of the smaller containers. Three groups of each species were required, since the plan was to examine one lot at the end of each of three growing seasons, and it was necessary to destroy the plants in order to examine the individual roots. Also three other smaller containers were planted with seed of the tenth species. In addition, 5 more of the smaller containers were used since it was desired to grow additional plants of certain species. Proper watering and mulching promoted prompt germination and good establishment. Once established, the seedlings were thinned to 5 or fewer in each planting.

Banding

In order to ascertain with certainty the longevity of the roots, a method of banding them individually was devised. Preliminary banding was done in 1929 and methods of procedure outlined. These were successfully used by Stoddart (4) in 1932. An opportunity to band roots on a large scale was afforded in the spring of 1943. Bands 8 to 10 millimeters long and 2 to 3 millimeters wide were cut from tin obtained from new, unpainted tubes such as are used for tooth pastes, ointments, etc. The thickness of the sheet of tin was only 0.12 of a millimeter, and hence it was very pliable yet durable. Bands remained bright and unrusted even after 3 years in the soil.

The first banding of the roots was done between April 26 and May 14, 1943, when the seedling grasses were 53 to 67 days old. The roots were exposed by removing the extension and washing away the soil by means of a gentle spray of water. It was usually necessary to support the tops of the plants by fastening them to a wire thrust deeply into the soil. Banding was done on damp or rainy days in much subdued light (5 to 10 per cent.), and over a wet floor. Care was taken to keep the roots moist by frequent spraying with a bulb type of hand spray. The grasses were uninjured by this root exposure. At the initial banding all roots 2 inches or more in length (excluding the seminal root or roots) were banded. Usually 2 to 6

roots, varying with the species, were too short to band. The number banded varied from 5 to 74 depending upon the species and the number of seedlings employed. The banding required two persons, one to separate the individual roots one by one from the others. This was done with a hand ice pick. The other person placed a band, which had previously been formed by rolling the tin into an open cylinder around the small end of a pipette, about the root by means of tweezers. It was then gently but tightly rolled between thumb and forefinger until it fit closely around the root (figs. 1, 2). The band partly unrolled if the root grew in diameter. When all the roots were banded, the extension was replaced and the roots covered with a mixture of

FIG. 1. Roots of switch grass (*Panicum virgatum*) about 3 months old, exposed for banding on June 11, 1943.

dry sand and soil. This readily filled all the spaces about them. It was added gradually and immediately watered by means of a bulb spray. This was repeated until the extension was filled.

A second banding, involving new roots of the same plants that developed later, was made between June 7 and 15, 1943. This was for the purpose of increasing the number of roots under observation. The plants had grown rapidly, tillered abundantly, and some had produced rhizomes. They were 12 to 21 inches tall. A few of the early bloomers had produced flower stalks, and some were flowering. After the extension was removed, a fine, gentle spray of water was used to slowly wash away the soil from the root-mass. Usually all of the new roots were banded, but in some species they were so numerous that only a part of them was used. The total number banded

per species varied between 100 and 419. In plants of most species the new roots formed a peripheral cover which obscured those banded earlier. After banding was completed, a process which required from 30 to 60 minutes or more, dry soil mixed with sand was placed about the roots and immediately watered. Even these larger plants showed no wilting or other signs of injury as a result of the root exposure. It was observed that with several species nothing was gained by banding before the new roots had enlarged considerably and that the same purpose could be accomplished by one banding at a date intermediate to those previously used. Hence, a single

FIG. 2. Roots of crested wheat grass (*Agropyron cristatum*) with numerous bands attached but not yet tightly rolled. It produces an abundance of roots early in life.

banding was made thereafter for plants that were grown to supplement the group employed at the beginning of the experiment.

In 1943, 2,551 roots of 107 plants were banded. The next year roots of several species of grasses were banded where they were growing in the prairie. The following year other lots were banded to ascertain the effect of frequent removal of tops on the longevity of roots.

CONDITIONS FOR GROWTH

During the growing season the plants were placed in a greenhouse with panels of glass removed from the sides and with windows and ventilators open. The cement floor was well insulated with a thick layer of dried grass, tubs of water were placed on side benches, and humidity was approximately the same as out-of-doors. The sides of the containers were also well insulated against direct sunlight. Although the light was only 50 to 60 per cent.,

the plants were widely spaced, and all made an excellent growth. All but two species, whose normal time for blossoming is in June, flowered the first season, and all flowered profusely each of the following summers. In autumn, the plants were clipped at a height of 2 inches (as is a mowed prairie), and the containers were placed out-of-doors in a large trench. The bottom of the trench was filled with gravel to furnish good drainage, and moist soil was firmly packed about the containers. The tops, now level with the soil, were lightly mulched with grass as is commonly furnished by the growth following fall mowing in prairie. The soil froze solidly to a depth of several inches each winter and in spring thawed and froze repeatedly. Each spring when growth began the containers were removed to the greenhouse until fall. This was done in order to protect the plants from injury by hail or by grasshoppers or other animals. Here proper care could best be given and especially insurance against waterlogging of the soil during periods of heavy rainfall. Moreover, injury by insects and competition by invading grasses could be more readily detected and prevented. Finally, the roots were much more easily recovered for final examination where each container could be moved about.

Examination of banded roots

In examining the banded roots, the container was placed on a small stout table about 18 inches high, so that it was accessible from all sides. The extension was removed after tapping it repeatedly to free it from the soil. It was sometimes necessary to insert a long-bladed knife between it and the soil. The tops of the plants were then removed at the soil surface. With a gentle stream of water, the mixture of sand and loam was slowly washed away, except for that portion close to the plants. A sharp, long-bladed knife was then used to cut through the soil mass and sever the roots at the level of the top of the container. It was completely loosened from the loam soil below. The top 4 inches of the root system with soil intact about it was then inverted and placed in a white enameled tray 18 by 15 inches in length and width and 2.25 inches deep. The tray was tilted, and the soil below the depth of banding was carefully washed away with a bulb spray or a very gentle stream from a hose.

As the tangled root-ends were exposed, they were removed with a scissors and saved for inspection. Muddy water and loose soil were caught in a tub as they left the tray. Soon the small glistening bands became visible (fig. 3). As soon as one was found the root within it was examined to ascertain whether or not it was still alive. While no completely satisfactory test has been devised to determine whether roots of grasses are living or dead, long experience with the roots of many individuals of each species adds greatly to the accuracy of determination. Living roots often had a yellowish-white or brownish color which was usually very different from the color of the dead ones. They had good tensile strength and were not brittle. The dead roots were easily broken, and tensile strength was low. They were often

smaller, and sometimes they had decayed, and the band lay free in the mass of roots. Once familiar with the roots of these grasses, dead roots could be distinguished with certainty where, as here, several inches of the roots were exposed.

After all the bands were removed from the more distal parts of the roots, two or more inches of the root-ends were again clipped. These were retained with the other clippings. Gentle spraying of the inverted roots revealed the bands near their proximal ends. They too were removed one by one. It was often necessary in older roots to carefully pry the crown apart to regain the last few bands. A record was kept of the total number of bands,

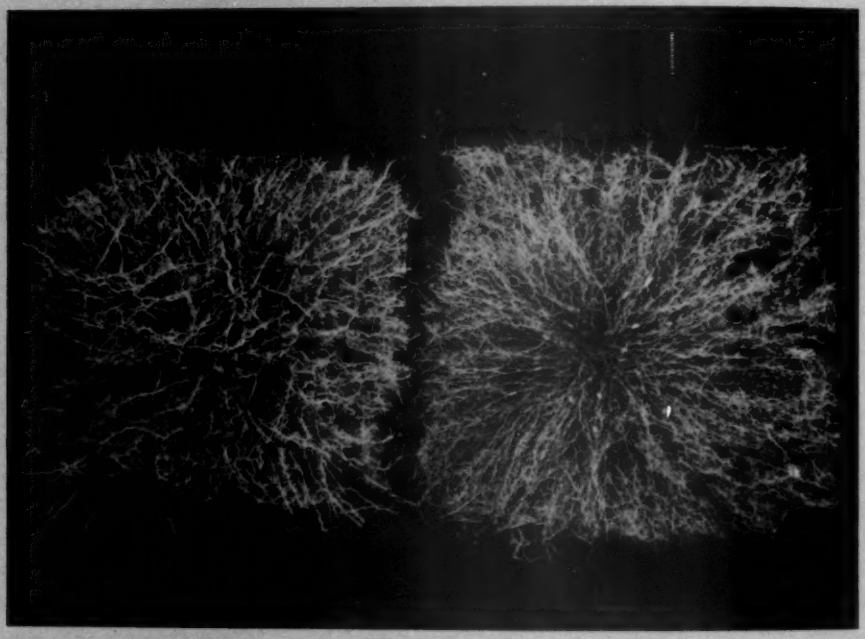


FIG. 3. (Left) Inverted mass of roots of 3 plants of *Panicum virgatum* that were cut from the top of a container after the extension had been removed. The soil has been partly washed from the roots, but they have not been cut back far enough to reveal the bands. (Right) Roots of *Andropogon scoparius*. The finer and more flexible roots of the 3 plants of this species have been pressed back and reveal some of the numerous bands. Both grasses were examined at the end of the second growing season. Each mass of roots is about 9 × 9 inches in diameter.

the number from living roots, and the number from dead ones as well as unattached bands. Since all of the washing was done in the white enameled tray and all soil was plainly visible in a very thin layer, it was only rarely that a band was washed out of the tray. Wash water and all soil were retained, however, until the total number of bands was accounted for.

Results

RESULTS OF FIRST GROWING SEASON AND FIRST YEAR

The number of banded roots of each species and the percentage that died either by the end of the first growing season or by the end of the first year

after banding are shown in table I. Each entry represents a separate group of plants, and where there is only one banding it indicates that new lots of plants were grown during a later season in order to increase the total number of banded roots.

Of the 7 species examined at the end of the first summer, only three showed root losses. These were *Bromus inermis* with 25 per cent. loss in one lot only. Losses in *Agropyron cristatum* were very low, 2 and 5 per cent., but *Stipa spartea* lost 18 per cent. Of roots examined at the end of the first year those of *Bouteloua curtipendula* alone suffered no fatalities; losses in four other species ranged from 16 to 36 per cent. (table I).

TABLE I

RESULTS OF FIRST GROWING SEASON AND FIRST YEAR

SPECIES*	NUMBER OF ROOTS BANDED		PERCENTAGE OF DEAD BANDED ROOTS	
	FIRST BANDING	SECOND BANDING	FALL, 1943	SPRING, 1944
Andropogon furcatus Muhl. (Big bluestem)	5	13	0	.....
	.....	60	0	.....
Elymus canadensis L. (Nodding wild rye)	26	43	.....	23
Panicum virgatum L. (Switch grass)	23	77	0	.....
Agropyron smithii Rydb. (Western wheat grass)	22	78	.....	36
Andropogon scoparius Michx. (Little bluestem)	11	45	0	.....
	.....	55	0	.....
Bouteloua curtipendula (Michx.) Torr. (Side-oats grama)	29	78	.....	0
Stipa spartea Trin. (Needle grass)	.....	33	18	.....
Bouteloua gracilis (H.B.K.) Lag. (Blue grama)	37	68	0	.....
Agropyron cristatum (L.) Beauv. (Crested wheat grass)	.....	56	2	.....
	.....	60	5	.....
	19	56	.....	25
Bromus inermis Leyss. (Hugarian brome grass)	33	69	25	.....
	.....	80	0	.....
	.....	60	0	.....
	34	66	.....	16

* The first three are tall grasses, the next four are mid grasses, blue grama is a short grass, and the last two are important cultivated species.

The excellent development of both roots and tops of grasses is shown in figure 4. *Bromus inermis* and *Andropogon scoparius*, both of which produced abundant flower stalks, had developed root systems nearly 3 feet long. There were 57 and 93 roots per plant, respectively. Thus, the mass of roots of the several seedlings in the clump was very large. This reflected the good conditions for growth.

RESULTS OF SECOND GROWING SEASON AND SECOND YEAR

The number of banded roots and the percentage of those that died by

fall, or by the following spring of the second year, are shown in table II. The plants were reduced to 9 species, since one lot of *Agropyron cristatum* grew very poorly. By the end of the second summer banded roots of *Andro-*

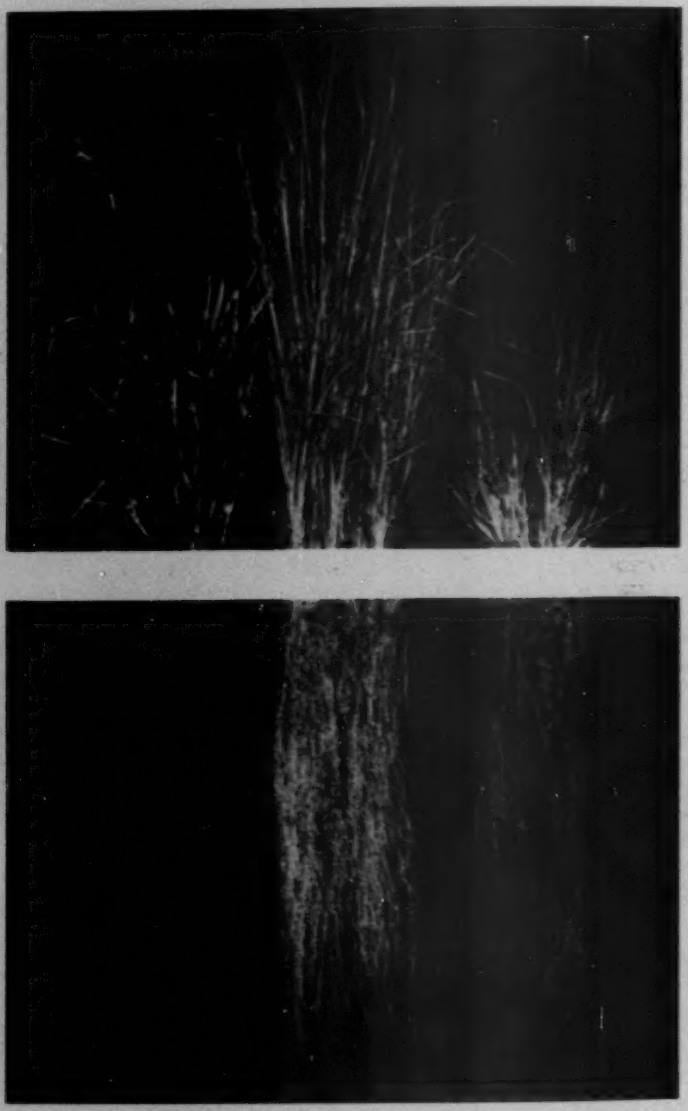


FIG. 4. Tops and deeper roots of one season's growth of *Bromus inermis* (left), *Andropogon scoparius*, and *Stipa spartea* (right). The top 4 inches of the root systems was destroyed in recovering the bands. Depth of root penetration was 32 inches.

pogon furcatus and *Panicum virgatum* had suffered no loss and those of *Andropogon scoparius* only 5 per cent. loss. But half or more than half of the banded roots of four other species died. The following spring loss in *Andropogon scoparius* was 31 per cent., which was nearly the same as

TABLE II

RESULTS OF SECOND GROWING SEASON AND SECOND YEAR

SPECIES	NUMBER OF ROOTS BANDED		PERCENTAGE OF DEAD BANDED ROOTS	
	FIRST BANDING	SECOND BANDING	FALL, 1944	SPRING, 1945
Andropogon furcatus	13	10	0	....
Elymus canadensis	35	67	....	73
Panicum virgatum	20	31	0	....
Agropyron smithii	20	47	55	....
	26	59	60	....
Andropogon scoparius	15	53	5	....
	31	69	....	31
Bouteloua curtipendula	33	68	....	64
Stipa spartea	17	23	50	....
Bouteloua gracilis	27	89	....	34
Bromus inermis	21	56	64	....

that of *Bouteloua gracilis*. In *Elymus canadensis* and *Bouteloua curtipendula* the loss was two-thirds or more of the roots banded.

RESULTS OF THE THIRD GROWING SEASON

Six species of grasses continued growth during the third summer. Plants of *Panicum virgatum* and *Agropyron cristatum* were accidentally destroyed. Work on *Bromus inermis* was terminated in the fall of 1944, and *Agropyron smithii* spread so widely in the container that it was necessary to remove it in the fall of 1944 in order to save *Stipa spartea* with which it was planted. The losses of banded roots among the six remaining species are shown in table III. They were lowest in *Andropogon furcatus* (19 per cent.), 55 per cent. in *Bouteloua gracilis*, and in four species they varied between 86 and 100 per cent.

SUMMARY OF SURVIVAL

The average percentage of survival of each of the 10 species at each period they were examined is shown in table IV. Losses in all species, except

TABLE III

RESULTS OF THIRD GROWING SEASON

SPECIES	NUMBER OF ROOTS BANDED		PERCENTAGE DEAD BANDED ROOTS
	FIRST BANDING	SECOND BANDING	FALL, 1945
Andropogon furcatus	20	60	19
Elymus canadensis	17	43	100
Andropogon scoparius	43	57	90
Bouteloua curtipendula	74	56	86
Stipa spartea	12	15	90
Bouteloua gracilis	45	45	55

TABLE IV

PERCENTAGE SURVIVAL AT EACH EXAMINATION

SPECIES	FALL, 1943	SPRING, 1944	FALL, 1944	SPRING, 1945	FALL, 1945
Andropogon furcatus	100	----	100	----	81
Elymus canadensis	----	77	----	27	0
Panicum virgatum	100	----	100	----	----
Agropyron smithii	----	64	42	----	----
Andropogon scoparius	100	----	95	69	10
Bouteloua curtipendula	----	100	----	36	14
Stipa spartea	82	----	50	----	10
Bouteloua gracilis	100	----	----	66	45
Agropyron cristatum	97	75	----	----	----
Bromus inermis	92	84	36	----	----

Agropyron cristatum, were gradual. *Agropyron smithii* had a survival at the end of the second summer of 42 per cent., and *Bromus inermis* of 36, but all banded roots were alive on *Panicum virgatum*. Percentage survival at the end of the third growing season was: *Andropogon furcatus* 81, *Bouteloua gracilis* 45, *B. curtipendula* 14, *Andropogon scoparius* 10, *Stipa spartea* 10, but on *Elymus canadensis* none survived.

NUMBER OF ROOTS PRODUCED

It is common knowledge that each spring new roots are produced, often in abundance, by perennial grasses, at least in temperate climates. It is important to consider the total number of roots in relation to the death of those produced early in life and the significance of the loss of the latter to the general welfare of the plant. Consequently, when the plants were examined a count of the total number of roots was made, and the average number per plant at different ages was thus ascertained (table V). All counts were of living roots; very few dead ones were found. In general, there was a steady increase in numbers with age. The number per plant was quite large and especially so in *Bromus inermis* (841). The very fine roots of *Bouteloua gracilis* likewise became very numerous (567), but the large, coarse ones of

TABLE V

AVERAGE NUMBER OF ROOTS PER PLANT AT DIFFERENT AGES

SPECIES	FALL, 1943	SPRING, 1944	FALL, 1944	SPRING, 1945	FALL, 1945
Andropogon furcatus	40	----	228	----	882
Elymus canadensis	307	----	----	319	438
Panicum virgatum	60	----	269	----	----
Agropyron smithii	----	249	----	----	----
Andropogon scoparius	69	----	248	323	177
Bouteloua curtipendula	----	170	----	423	378
Stipa spartea	24	----	----	----	175
Bouteloua gracilis	37	----	----	167	567
Agropyron cristatum	----	329	----	----	----
Bromus inermis	----	841	794	----	----

Andropogon furcatus were even more abundant (882). A single clump of 4 seedlings of *Bouteloua gracilis* produced during three seasons of growth a total of 2,268 individual roots (fig. 5).

In the examination of three-year-old bunches of *Bouteloua curtipendula* it was observed that a few dead rhizomes occurred in the center of the clump.

FIG. 5. Bunches of *Bouteloua gracilis* (left) and *B. curtipendula* at the end of the third growing season. In the root system of the first (cut off at a depth of 16 inches) roots occurred at the average rate of 28 per square inch. The four plants in the bunch on the right have a total of 1,512 roots.

This was only 1 to 3 per cent. of the total interwoven lot. Likewise a part of the central and older portion of the crown of *Andropogon scoparius* had died. This, while very small, was the part to which most of the older roots (mostly banded) had been attached. In prairie, degeneration of plants of these species and of many bunch grasses usually begins in the center

of the crown and proceeds outward. Such degeneration would be well advanced in the soil before it would be at all conspicuous above ground, due to the crowding of stem and leaves. This may have been the beginning of the process. Certainly the loss of a few score of roots among hundreds of others would have little effect upon the plant. The writers have determined experimentally that under usual conditions of growth the removal of half or even more of the root system had little harmful effect upon the growth of several species of grasses. Whether or not the roots that develop the second and third years are of longer life-span than those of the first remains to be ascertained.

Root banding in prairie

The roots of six species of grasses were banded where they grew in clay loam soil in prairie. This was accomplished with much more difficulty than when the plants were grown in containers. Early in June, 1944, the soil was removed by means of a continuous stream of water from a portable spray pump to a depth of 3 to 4 inches about the roots of four species of bunch-forming grasses. Trenches 18 inches deep were dug in the sod of *Andropogon furcatus* and *Sorghastrum nutans* (L.) Nash. By using the spray of water on a wall of the trench, numerous roots were exposed. A few, as in the bunches, were white and turgid and clearly of the current year's growth. Most of them were one or more years old. The exact age, of course, could not be determined. While the roots were kept moist by frequent sprinkling, the bands were attached to selected uninjured individuals. Moist soil was replaced about them and thoroughly watered. The exact location of the plants was carefully marked by stakes driven into the soil so that the roots could be recovered later by removing a block of sod of minimum size.

In April of the following spring bands were recovered by the washing process already described. This was far more difficult than with sand-loam soil even after the sods were thoroughly soaked in water. Four of the 20 banded roots of *Stipa spartea* were dead, but only 1 among 24 roots of *Adropogon scoparius*. On young bunches of June grass (*Koeleria cristata* (L.)) Pers., 6 of the 30 banded roots had died, but 5 among 14 on mature plants.

The remaining bands were recovered from the prairie at the end of the second summer. The number varied from 30 to 60 per species. Losses in one bunch of *Stipa spartea* were 54 per cent., in another 60. Other losses were: *Andropogon scoparius* 23 per cent., *A. furcatus* 45, *Bouteloua curtipendula* 36, and *Sorghastrum nutans* 37. This work was done primarily to ascertain whether or not the method was feasible under field conditions; the losses may or may not be representative of those over a long period of time.

Effect of clipping of tops on longevity of roots

The banding method was used in an experiment on the length of life of both seedlings and established plants of several species of grasses. Seeds of

Bromus inermis were planted in the large type of container previously described. When the seedlings were 6 inches tall and tillered abundantly (May 10) 130 roots were banded. A week later the plants were clipped at a height of 2 inches (to simulate grazing) and every 10 days thereafter until June 20. After each clipping recovery occurred rather promptly. When the roots were examined on August 2 the plants were 5 to 10 inches tall and in good condition. Only 20 of the banded roots had died, which was 15 per cent. of those originally marked. On the unclipped controls all of the 140 banded roots remained uninjured.

A similar experiment was performed at the same time with *Agropyron cristatum*. It recovered less promptly after clipping and grew poorly. Hence, next to the last clipping was omitted. On August 2 many of the tops below the clipping level were partially dead and dry, but all of the plants had made some new growth, which was 3 to 6 inches tall. Of the 110 roots banded 80 had died. Of the unclipped controls, only 3 per cent. of the 116 banded roots had succumbed. Thus, repeated clipping resulted in a mortality of 73 per cent. of the roots.

Four pieces of sod containing vigorous plants of *Andropogon furcatus*, and 4 with *A. scoparius*, were secured on April 15 from a prairie where the plants were just renewing growth. Each block of sod was 5 inches long, 3 inches wide, and 3.5 inches deep. Each was placed in a separate box on a layer of moist sand and loam 5 inches deep, and soil was compacted about it. The tops grew rapidly and by May 17 abundant new roots 4 to 8 inches long had developed. The soil was now washed from the roots extending below the blocks of sod, the roots banded, and the sods carefully transplanted in the large containers previously used. They contained a mixture of sand and loam which was placed about the roots and sods in a manner that did them no injury.

A total of 53 bands was placed on the new roots of the two lots of plants used as controls and 60 on those of the plants to be clipped. The experimental plants were clipped at a height of 2 inches and the controls at 4 inches immediately after this second transplanting. Clipping, except for the controls, was repeated at intervals of 10 to 14 days until July 8, a total of 5 clippings. The plants recovered promptly after each clipping except the last, *Andropogon furcatus* showing better growth than *A. scoparius*. When examined on August 1, only 3 per cent. of the roots of the controls of *Andropogon furcatus* had died but 45 per cent. of those of the clipped plants. In *A. scoparius* the percentages of death of control and clipped were 3 and 64, respectively. Thus, the injurious effects of the frequent removal of most of the tops is shown by the response of roots as well as by death in the crown, a loss that could be definitely ascertained by the banding method.

Discussion

The banding method was found unsatisfactory for some species of grasses with very fine roots. In *Poa pratensis*, *Eragrostis trichodes*, and a few other

species it was difficult to adjust the bands tightly enough to keep other roots from entering the same band. This difficulty was not encountered with most of the native prairie grasses. Also where the number of moderately fine roots was very great and the roots compact, as in *Bromus inermis* and *Agropyron cristatum*, banding was more difficult. That the process of banding or the presence of the bands did not harm the roots is apparent since in most plants all of the banded roots were alive at the end of the first summer and many after a year or more.

Bands were much more readily recovered from bunch grasses than from those producing sods, although certain sod-formers retained the bunch habit for a long time. *Agropyron smithii*, for example, had only a few fine roots

FIG. 6. The first four inches of underground parts of *Agropyron smithii*. The large, widely spreading, white rhizomes are more prominent than the rather small fine roots. This made banding difficult as well as the replacement of the soil without root injury. June 14, 1942.

that supported the seedling plants. Very early several slender rootstocks developed which produced whorls of a few roots each from the nodes, which were an inch or more apart. Hence, the area for banding soon equaled the area of the soil surface (fig. 6). Quite in contrast were the root habits of bunch formers, and even *Andropogon furcatus* and *Panicum virgatum* in early life. They took complete possession of the soil immediately below the crown. The roots of these two species became so large (often two or more millimeters in diameter), even after two or three months, that they were closely compacted at their points of origin from the lower nodes and abundant tillers.

A close check was had on root losses from the two species of Andropogon by the minute examination of the roots of about 50 plants of each species each fall. They were grown out-of-doors in large steel drums for experimental purposes. In preparing these washed roots for weighing, the crowns were separated, and from each portion of the crown the roots were cut individually or in such small groups that every root was distinctly visible. They were then finally washed in a white enameled tray where dead or discolored roots could be seen plainly. None was observed at the end of either the first or second growing season. They occurred occasionally the third year.

No losses and replacements of root systems similar to those reported by SPRAGUE (3) and STUCKEY (5) occurred. Sprague examined plants whose roots were almost confined to the first 9 inches of a gray-brown forest soil in New Jersey. He found that one-half of the root system, by weight, was newly generated each year. Stuckey states that for some of the species the whole root system was regenerated annually, with active production of new growth beginning in October. Most of the old roots degenerated shortly after the new ones developed. These species included *Phleum pratense* L., *Festuca elatior* L., *Poa trivialis* L., *Lolium perenne* L., and probably *Agrostis tenuis* Sibth. and *Agrostis alba* L. But on *Poa pratensis* L., *Poa compressa* L., *Agropyron cristatum* (L.) Beauv. and *Dactylis glomerata* L., only a small percentage of roots disintegrated the first year. Although data by STODDART (4) are meager the results with each of the four species of prairie grasses banded are in accord with those of the writers.

Field observations over a long period of years indicate that *Elymus canadensis*, a grass common in moist places, is a species that depends for its permanency of occupation more upon rapid reproduction from seed than upon length of life of the individual. The limited data on *Bromus inermis* and *Agropyron cristatum* indicate considerable permanence of the roots developed the first year. All of the other prairie grasses grown are species of great stability, some individuals probably enduring for a quarter-century or more. While there was a gradual loss of the older roots year by year, the loss is indeed small if not entirely negligible when the great abundance of new roots produced annually is considered. For example, the loss in *Bouteloua gracilis* of 90 per cent. of the roots it produced by June 10 of the first summer amounted after three growing seasons to only 8 per cent. of the total living roots. Similar losses in *Andropogon furcatus* were 2 per cent.

Long life and hence permanency of occupation are characteristics which aid greatly in the adaptation of a grass to semiarid and arid climates. They are likewise important characters of grasses well adapted to soil binding and prevention of erosion.

Summary

Seeds of 10 species of perennial range and pasture grasses were planted in triplicate lots in loam soil in containers large enough for ample root

development. A removable extension at the top of each container was filled with sandy loam soil easily washed away when the extension was removed, thus exposing the roots for examination.

Small bands of very thin, pliable sheet tin were placed around individual roots at each of two stages in the development of the plant. A total of 3,424 roots of 181 plants were banded to ascertain their longevity.

The roots were kept moist while exposed and then covered with dry soil which was immediately watered. Some were examined at the end of the first and second year, respectively, and the remainder at the end of each of three growing seasons.

Ninety-seven per cent. of the banded roots of *Agropyron cristatum* survived the first summer and 75 per cent. the first year. Survival on *Bromus inermis* was 92, 84, and 36 per cent. at three examinations. Survival on *Panicum virgatum* and *Agropyron smithii* was, after the second summer, 100 and 42 per cent., respectively. After three growing seasons 81 per cent. of the roots on *Andropogon furcatus* survived, but none on *Elymus canadensis*. Losses in all species were gradual, and after three growing seasons survival of roots was as follows: *Bouteloua gracilis* 45 per cent., *B. curtipendula* 14, and *Andropogon scoparius* and *Stipa spartea* each 10 per cent.

Average number of roots produced by individual plants varied from 175 to 882 at the end of the third summer. Compared with the total number of roots, losses among the banded roots were small to negligible. They often amounted to only 2 to 8 per cent. of the total number of living roots. Nearly all of the species studied have a long life and show great permanency of occupation, both important features in prevention of soil erosion.

Roots of 6 species of grasses of unknown age were banded in clay loam soil in prairie. At the end of a second growing season, losses ranged from 23 to 45 per cent. except in *Stipa spartea*, where they were 57.

Established seedlings of *Bromus inermis* and *Agropyron cristatum* with banded roots were clipped at 2 inches' height at 10-day intervals. There was no loss from unclipped control plants of the first species and only 3 per cent. from the second. But roots of clipped Bromus suffered a loss of 15 per cent. and those of Agropyron 73.

New roots from vigorous transplanted blocks of sod of *Andropogon furcatus* and *A. scoparius* were banded and the tops of one lot of each clipped at intervals of 10 to 14 days. After 5 clippings losses of roots were 45 and 64 per cent., respectively. The unclipped controls lost only 3 per cent. each.

THE UNIVERSITY OF NEBRASKA
LINCOLN, NEBRASKA

LITERATURE CITED

1. PAVLYCHENKO, T. Root systems of certain forage crops in relation to the management of agricultural soils. Nat. Res. Council of Canada and Dom. Dept. Agr. N. R. C. No. 1088. 1942.

2. SHIVELY, S. B., and WEAVER, J. E. Amount of underground plant materials in different grassland climates. Univ. Nebraska Cons. and Surv. Div. Bul. **21**. 1939.
3. SPRAGUE, H. B. Root development of perennial grasses and its relation to soil conditions. Soil Sci. **36**: 189–209. 1933.
4. STODDART, L. A. How long do roots of grasses live? Science n.s. **81**: 544. 1935.
5. STUCKEY, I. H. Seasonal growth of grass roots. Amer. Jour. Bot. **28**: 486–491. 1941.
6. WEAVER, J. E. Ecological relations of roots. Carnegie Inst. Washington Pub. No. **286**. 1919.
7. ———. Root development in the grassland formation. Carnegie Inst. Washington Pub. No. **292**. 1920.
8. ———, and HARMON, G. W. Quantity of living plant materials in prairie soils in relation to run-off and soil erosion. Univ. of Nebraska Cons. and Surv. Div. Bul. **8**. 1935.
9. ———, HOUGEN, V. H., and WELDON, M. D. Relation of root distribution to organic matter in prairie soil. Bot. Gaz. **96**: 389–420. 1935.
10. ———, and ZINK, E. Extent and longevity of the seminal roots of certain grasses. Plant Physiol. **20**: 359–379. 1945.

EFFECTS OF POTASSIUM ON THE NITROGENOUS CONSTITUENTS OF *ANANAS COMOSUS* (L.) MERR.[1]

C. P. Sideris and H. Y. Young

(WITH ONE FIGURE)

General

Results obtained at various times from field experiments have shown that the nitrate content of the tissues of *Ananas comosus* may be increased and the time of floral differentiation delayed by high applications of potassium in the field. These findings, suggesting interrelationships between nitrogen, potassium, plant growth and floral differentiation, are of significance and deserve attention.

This study, concerned with the nitrogenous constituents of the tissues of *A. comosus* (L.) Merr. grown in nutrient solutions containing either high or low amounts of potassium and supplied with equal quantities of nitrogen either as nitrate or ammonium, is intended to furnish information on potassium and nitrogen interrelationships in growth. Ash constituents, chlorophyllose pigments, titratable acidity, and carbohydrates in the same plants were reported in earlier publications (**20, 21**). The effects of nitrate and ammonium nutrition on the nitrogenous fractions of *A. comosus* were also reported previously (**22**), but without variation in potassium.

Review of literature

Potassium-deficient plants may contain in some organs higher amounts of soluble organic nitrogen and lower amounts of protein nitrogen than plants supplied with ample or luxury amounts of potassium. This condition has been variously explained by different investigators. Richards and Templeman (**13**) state that K-deficiency (a) causes rapid disappearance of protein in old but not in new leaves; (b) increases the amounts of amide- and amino-nitrogen; and (c) promotes accumulation of nitrate. Wall (**30, 31, 32**) claims that K-deficiency curtails protein synthesis at the transitional amino acid to protein stage. Burrell (**1**) observed that K-deficiency in soybean plants increased slightly the amounts of amino- and amide-nitrogen but decreased protein nitrogen. Nightingale *et al.* (**8**) have shown that in K-deficient tomato plants the amounts of soluble organic nitrogen increased while those of nitrate- and protein-nitrogen decreased. Schmalfuss (**17**) claims that K-deficient plants of *Avena sativa* and *Tradescantia laikenensis* contained higher values of dry matter and soluble organic nitrogen and lower of protein nitrogen than K-supplied plants. The same author also reports that the proportion of soluble- to protein-nitrogen depends on whether

[1] Published with the approval of the Director as Technical Paper no. 162 of the Pineapple Research Institute, University of Hawaii.

the nitrogen in the nutrient solution is supplied as nitrate or ammonium. TURTSCHIN (**28**) is of the opinion that K-deficient plants are unable to utilize nitrogen as ammonium because of the accumulation of toxic concentrations of NH_4-ions and of glucose. HIBBARD and GRIGSBY (**7**) have found no evidence that increases in potassium or calcium parallels the amounts of nitrogen and protein. HARTT (**6**) claims that in the blades of 7-month sugar cane plants higher percentages of amino-, protein-, and total-nitrogen were found in potassium-deficient than potassium-supplied cultures, while in the stems the opposite relationship was found to occur. PHILLIPS *et al.* (**9, 10, 11**) found more solids, reducing sugars, and insoluble nitrogen in K-deficient than in K-supplied plants. ROHDE (**14**) states that a balance between potassium and nitrogen supply is necessary for efficiency in the assimilation of CO_2 and use of available light. GREGORY (**2**) has summarized his views as follows: "Potassium deficiency is characterized by (a) a marked increase in amino- and amide-nitrogen; (b) accumulation of nitrate nitrogen in later leaves; and (c) a very rapid breakdown of protein during senescence of leaves. At emergence the leaves show a normal protein content. It is held that potassium is not primarily associated with protein synthesis but is necessary for maintaining the protoplasmic complex, and in its absence rapid proteolysis occurs. In consequence of this protein breakdown, soluble nitrogen fractions accumulate throughout the plant."

Methods

The composition of the nutrient solutions, cultural procedures, methods of chemical analyses of the tissues and of statistical treatment of the data have been described in previous publications (**19, 20, 22**).

The titles of the treatments are reported synoptically in the text. Thus the 205 and 4 mg. of potassium per liter are designated by High-K or high potassium, and Low-K or low potassium, and the nitrate- and ammonium-nitrogen series by N-n and A-n, respectively.

Observations

TOTAL NITROGEN

Total nitrogen, expressed in table II as the sum of the inorganic and organic nitrogen fractions, was higher in the High-K than Low-K cultures, being 1.162 and 1.026 times greater for the N-n and A-n series, respectively. Relative concentration ratios (R.C.R.), obtained by dividing the nitrogen ratio of High-K to Low-K cultures by the respective plant weight ratios, indicate the relative concentrations of various nitrogenous fractions in the High-K cultures with reference to the Low-K cultures. Such ratio values in table II show that the concentrations of total-N in the leaves and stem of the High-K cultures were smaller than those of the Low-K cultures. Similar concentrations in the roots were lower for the High-K cultures in the N-n series but higher in the A-n series.

Inorganic nitrogen, the sum of $NH_4 + NO_3$, as percentage of total nitro-

gen was 6.10 and 2.50 per cent. in the N-n series and 0.90 and 0.32 per cent. in the A-n series for the High-K and Low-K cultures, respectively. The higher values of inorganic nitrogen in the N-n series resulted from nitrates which are not assimilated until reaching the chlorophyllose tissues of the leaves.

Total organic nitrogen

This fraction, expressed as the sum of soluble organic-N and protein-N, is reported in table I as mg. per gram of fresh tissue. The data show significant difference between High-K and Low-K cultures in the organic-N content of the leaves (odds 1 : 3000 for the N-n and 1 : 10,000 for the A-n series) and of the stem of the A-n series (odds 22 : 1) but not of the N-n series. Such differences in the leaves in favor of the Low-K cultures were 0.20 and 1.23 mg. per gram for the N-n and A-n series, respectively. The data in table II reporting total amounts of organic nitrogen per plant show that such amounts were higher in the High-K than Low-K cultures because of greater plant weights in the former than the latter. However, it is interesting to note that organic nitrogen values, obtainable from the sum of soluble organic-N and protein-N in table II, differed more between High-K and Low-K cultures in the N-n series (5,543 vs. 4,954 mg.) than in the A-n series (6,289 vs. 6,165 mg.). Such differences may suggest that in the N-n series nitrogen absorption as nitrate was greatly influenced by the concentrations of potassium in the nutrient solution whereas similar influences on ammonium absorption in the A-n series were negligible. Moreover, these findings indicate that NH_4 and K ions, although of the same electric charge, are not mutually antagonistic in their relationship to absorption by plants.

Relative concentration ratios of total organic-N, obtainable from one-half the sum of soluble organic-N and protein-N values in table II, were smaller for the High-K than Low-K cultures in both series of nitrogen types. Comparison of relative concentration ratio values as obtained above between N-n and A-n series shows that leaf and stem, but not root values, were lower in the latter than former series.

Soluble organic nitrogen

Soluble organic nitrogen values, reported in table I and figure 1, were higher in the Low-K than High-K cultures. In the leaves the difference of the means of soluble organic-N between High-K and Low-K cultures in favor of the latter cultures, was 0.15 and 0.99 mg. per gram for the N-n and A-n series, respectively. Similar differences in the stem for soluble organic-N, also in favor of the Low-K cultures, were 0.38 and 1.33 mg. per gram for the N-n and A-n series, respectively. Soluble organic-N values per plant, reported in table II, were approximately the same (2,380 vs. 2,294 mg.) in both High-K and Low-K cultures in the N-n series, but in the A-n series those of the Low-K cultures were much greater than of the High-K cultures (3,560 vs. 2,753 mg.). Relative concentration ratio values were generally lower in the High-K than Low-K cultures in both series.

TABLE I

EFFECTS OF 205 VS. 4 MG. PER LITER OF POTASSIUM SUPPLIED TO SOLUTION CULTURES CONTAINING EQUAL QUANTITIES OF EITHER NITRATE-N OR AMMONIUM-N ON THE AMOUNTS (MG. PER GM. OF FRESH

TABLE 1

EFFECTS OF 205 VS. 4 MG. PER LITER OF POTASSIUM SUPPLIED TO SOLUTION CULTURES CONTAINING EQUAL QUANTITIES OF EITHER NITRATE-N OR AMMONIUM-N ON THE AMOUNTS (MG. PER GM. OF FRESH TISSUE) OF TOTAL ORGANIC-N, SOLUBLE ORGANIC-N AND PROTEIN-N IN DIFFERENT SECTIONS OF THE LEAVES, STEM, AND ROOTS OF ONE-YEAR-OLD *Ananas comosus* (L.) MERR.

PLANT SECTIONS	NITRATE-N						AMMONIUM-N					
	HIGH-K			LOW-K			HIGH-K			LOW-K		
	TOTAL ORG. N	SOL. ORG. N	PROT. N	TOTAL ORG. N	SOL. ORG. N	PROT. N	TOTAL ORG. N	SOL. ORG. N	PROT. N	TOTAL ORG. N	SOL. ORG. N	PROT. N
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Leaves												
Old (B) 1+2 (base)	1.21	0.43	0.78	0.97	0.35	0.62	0.96	0.23	0.73	1.82	0.90	0.92
3	1.38	0.56	0.82	1.20	0.37	0.83	1.26	0.29	0.97	2.41	1.47	0.94
4	1.69	0.81	0.88	1.69	0.53	1.16	1.93	0.69	1.24	3.58	1.85	1.73
5 (tip)	1.43	0.66	0.77	1.70	0.46	1.24	2.41	1.02	1.39	3.96	1.85	2.11
Mean	1.43	0.62	0.81	1.39	0.43	0.96	1.64	0.56	1.08	2.94	1.52	1.43
Mature (C) 1 (base)	1.10	0.26	0.84	0.92	0.50	0.42	0.61	0.15	0.46	1.65	0.93	0.72
2	1.07	0.29	0.78	1.06	0.47	0.59	0.63	0.09	0.54	1.91	1.47	0.44
3	1.59	0.65	0.94	1.74	0.79	0.95	1.40	0.52	0.88	3.23	1.73	1.50
4	1.80	0.79	1.01	2.28	1.12	1.16	2.14	0.87	1.27	4.38	2.38	2.00
5 (tip)	2.11	0.82	1.29	2.50	1.06	1.44	2.70	1.34	1.36	4.99	2.91	2.08
Mean	1.53	0.56	0.97	1.70	0.79	0.91	1.50	0.59	0.90	3.23	1.88	1.35
Active (D) 1 (base)	0.65	0.41	0.24	1.12	0.78	0.34	0.46	0.12	0.34	1.39	0.73	0.66
2	0.77	0.53	0.24	0.89	0.43	0.46	0.54	0.16	0.38	1.12	0.58	0.54
3	1.24	0.58	0.66	1.70	0.81	0.89	1.45	0.50	0.95	2.37	1.50	0.87
4	1.85	0.68	1.17	2.20	1.17	1.03	2.22	1.08	1.14	3.66	2.00	1.66
5 (tip)	2.20	0.97	1.23	2.70	1.45	1.25	3.00	1.40	1.60	4.93	3.47	1.46
Mean	1.34	0.63	0.71	1.72	0.93	0.79	1.53	0.65	0.88	2.69	1.66	1.04
Young (E) 1 (base)	1.20	0.59	0.61	1.45	0.71	0.74	1.10	0.71	0.39	1.69	1.08	0.61
2	0.79	0.33	0.46	0.93	0.47	0.46	0.74	0.33	0.41	1.27	0.77	0.50
3	1.23	0.56	0.67	1.41	0.66	0.75	1.28	0.52	0.76	1.90	1.26	0.64
4	1.66	0.72	0.94	2.40	1.16	1.24	2.02	0.86	1.16	3.32	2.03	1.29
Mean	1.22	0.55	0.67	1.55	0.75	0.80	1.28	0.60	0.68	2.04	1.28	0.76
Leaves, mean	1.39	0.59	0.80	1.59	0.74	0.87	1.49	0.60	0.88	2.72	1.59	1.15
Stem												
Base	1.91	0.80	1.11	1.74	0.77	0.97	1.38	0.60	0.78	1.82	1.15	0.67
Middle	2.01	0.80	1.21	2.17	1.28	0.89	1.14	0.60	0.54	2.63	2.23	0.40
Apex	1.92	0.70	1.22	2.35	1.39	0.96	1.46	0.90	0.56	3.02	2.70	0.32
Mean	1.95	0.77	1.18	2.09	1.15	0.94	1.33	0.70	0.63	2.49	2.03	0.46
Roots	1.16	0.20	0.96	1.46	0.32	1.14	1.27	0.35	0.92	1.07	0.35	0.72

TABLE II

EFFECTS OF "HIGH" VS. "LOW" (205 VS. 4 MG. PER LITER) POTASSIUM ON MEAN PLANT, LEAF, STEM AND ROOT WEIGHTS, AMOUNTS, RATIOS AND RELATIVE CONCENTRATION RATIOS (R.C.R.) OF TOTAL-N, SOLUBLE ORGANIC-N, PROTEIN-N, AMMONIUM-N AND NITRATE-N OF HIGH-K TO LOW-K CULTURES IN ONE-YEAR-OLD *A. comosus* GROWN IN SOLUTION CULTURES SUPPLIED WITH EQUAL AMOUNTS OF NITROGEN EITHER AS NITRATE OR AMMONIUM

ITEMS	UNITS OF MEASURE	NITRATE SERIES				AMMONIUM SERIES			
		HIGH-K	LOW-K	RATIO HK/LK	R.C.R. HK VS. LK	HIGH-K	LOW-K	RATIO HK/LK	R.C.R. HK VS. LK
Plant weights	gm.	3,855	2,745	1.405	.....	3,895	2,250	1.731	.....
Leaf	"	3,210	2,280	1.408	.....	3,330	1,820	1.830	.....
Stem	"	335	173	1.936	.....	342	120	2.850	.....
Root	"	314	288	1.090	.....	197	310	0.636	.....
Total-N	mg.								
Plant	"	5,903	5,082	1.162	0.826	6,346	6,184	1.026	0.593
Leaves	"	5,066	4,221	1.200	0.852	5,631	5,503	1.023	0.559
Stem	"	648	407	1.592	0.822	463	347	1.338	0.470
Roots	"	189	454	0.416	0.382	252	334	0.755	1.186
Sol. org.-N	mg.								
Plant	"	2,380	2,294	1.037	0.738	2,753	3,560	0.773	0.446
Leaves	"	2,063	1,998	1.030	0.732	2,437	3,212	0.759	0.415
Stem	"	254	204	1.245	0.643	247	239	1.033	0.362
Roots	"	63	92	0.685	0.628	69	109	0.633	0.994
Protein-N	mg.								
Plant	"	3,163	2,660	1.190	0.847	3,536	2,605	1.357	0.784
Leaves	"	2,764	2,139	1.292	0.916	3,146	2,275	1.383	0.756
Stem	"	303	191	1.586	0.820	209	107	1.953	0.684
Roots	"	96	330	0.291	0.267	181	223	0.813	1.278
Ammonium-N	mg.								
Plant	"	38	29	1.310	0.932	57	19	3.000	1.735
Leaves	"	31	24	1.290	0.916	48	16	3.000	1.640
Stem	"	4	2	2.000	1.034	7	1	7.000	2.455
Roots	"	3	3	1.000	0.918	2	2	1.000	1.572
Nitrate-N	mg.								
Plant	"	322	99	3.255	2.320	0	0	0	0
Leaves	"	208	60	3.465	2.460	0	0	0	0
Stem	"	87	10	8.700	4.500	0	0	0	0
Roots	"	27	29	0.930	0.853	0	0	0	0

Protein nitrogen

Mean values of leaf protein-N, reported in table I and figure 1, were higher for the Low-K than High-K cultures in both series. The difference of the means between High-K and Low-K cultures, in favor of the latter, was 0.07 and 0.27 mg. per gram of fresh tissue or 8.8 and 30.7 per cent. for the N-n and A-n series, respectively. Similar differences in the stem, but in favor of the High-K cultures, were 0.24 and 0.17 mg. per gram of fresh tissue or 25.5 and 37.0 per cent. for the N-n and A-n series, respectively.

Protein-N values of the leaves, reported in table II and figure 1, in favor of the High-K cultures, were 1.292 and 1.383 times greater for the N-n and A-n series, respectively. Similar values of the stems, also in favor of the

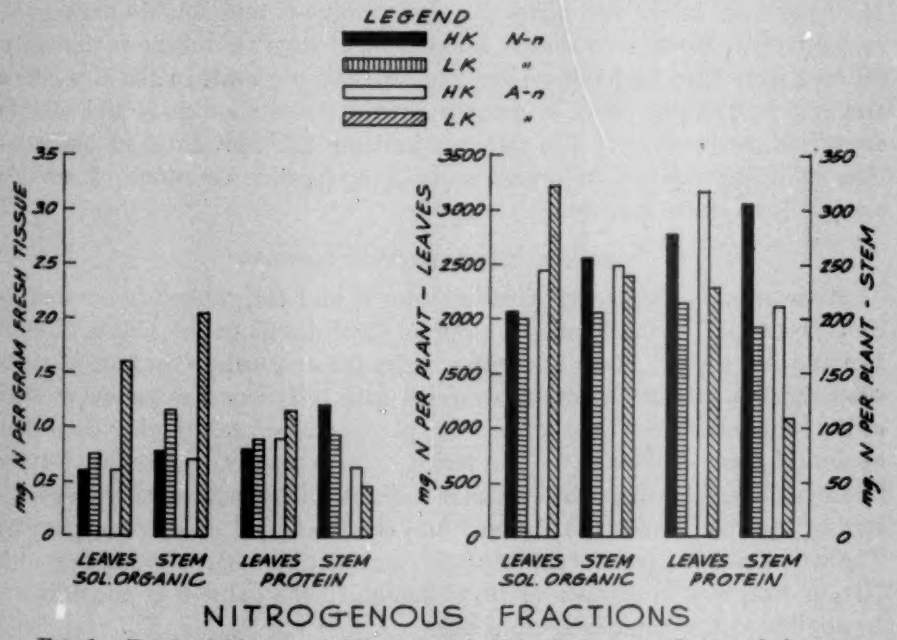


Fig. 1. Total soluble organic-N and protein-N in the leaves and stem of *A. comosus* grown in solution cultures with 205 p.p.m. of K (H-K) or with 4 p.p.m. (L-K) and either with nitrate (N-n) or ammonium (A-n) nitrogen.

High-K cultures, were 1.586 and 1.953 times greater for the N-n and A-n series, respectively. The data disclose that the rate of conversion of soluble organic-N to protein-N was greater in the stem than in the leaves of the High-K cultures, being 1.228 (1.586 ÷ 1.292) and 1.412 (1.953 ÷ 1.383) times in the N-n and A-n series, respectively. On the basis of information reported elsewhere (**22**), the higher protein-N and soluble organic-N values in the A-n than N-n series should be attributed to a greater rate of absorption by roots of NH_4 than NO_3 ions from the nutrient solution.

Relative concentration ratio values of protein-N, in table II, disclose that in leaves and stem such values were lower in the High-K than Low-K cultures. Similar values of the roots were likewise lower for the High-K than Low-K cultures in the N-n series but higher in the A-n series.

Alpha amino nitrogen

Alpha amino-N, a fraction of soluble organic-N determined by the Van Slyke (27) gasometric method, reported in table III, was higher in practically all plant sections of the Low-K than High-K cultures in both series. The difference of the means of alpha amino-N between High-K and Low-K cultures, in favor of the latter cultures, was in the leaves 30.2 and 145.6 per cent. and in the stem 21.6 and 82.5 per cent. for the N-n and A-n series, respectively.

Comparison of the above differences of alpha amino-N, in table III, to soluble organic-N, in table I shows that the leaf values were approximately the same for both; i.e., 30.2 vs. 28.3 per cent. in the N-n series and 165.0 vs. 145.7 per cent. in the A-n series for alpha amino-N and soluble organic-N, respectively. However, a similar comparison of stem values shows that they differed more than leaf values; i.e., 21.6 vs. 49.3 per cent. in the N-n series and 82.4 vs. 190.0 per cent. in the A-n series for alpha amino-N and soluble organic-N, respectively. The findings indicate different rates of assimilation or polymerization of alpha amino-N and other fractions of soluble organic-N in leaves and stem.

Inorganic nitrogenous fractions

Ammonium nitrogen, reported in tables II and III, ranged in concentrations from 0.005 to 0.019 mg. per gram of fresh tissue in the plants of both N-n and A-n series. Even in the A-n series the ammonium content of roots submerged in nutrient solutions supplied with NH_4 ions was as low as that of the terminal leaf sections of the same plants and slightly higher than that of similar plant sections in the N-n series. These results, supporting former findings (22), show that NH_4 ions were assimilated immediately after absorption by the root tissues and changed into compounds of greater complexity. The traces of NH_4 ions found in all sections of plants supplied either with NO_3 or NH_4 ions in solution cultures should, in the opinion of the authors, be attributed to deaminizing processes.

The amounts of nitrate-nitrogen, mostly present in the non-chlorophyllose sections of the leaves, stem, and roots of the N-n series were greater in the High-K than Low-K cultures. Both cultures in the A-n series completely lacked nitrate nitrogen, thus confirming previous findings (22) that nitrification in nutrient solutions is either very little or nil. Nitrate-N was from 1.1 to 4.9 times greater in the basal sections (no. 1) of the leaves of the High-K than Low-K cultures in the N-n series. Similar values of the stem were from 2.5 to 5.7 times higher in the High-K than in the Low-K cultures. Such comparisons between High-K and Low-K cultures indicate that anion absorption (NO_3) was greater from the High-K than Low-K cultures, presumably on account of higher concentrations of cations; i.e., K ions.

Discussion

Different concentrations of potassium in combination with different sources of inorganic nitrogen in nutrient solutions modified the relative

TABLE III

EFFECTS OF "HIGH" AND "LOW" AMOUNTS OF POTASSIUM (205 VS. 4 MG. PER LITER OF SOLUTION CULTURE) IN NUTRIENT SOLUTIONS SUPPLIED EITHER WITH NITRATE OR AMMONIUM SALTS

TABLE III

EFFECTS OF "HIGH" AND "LOW" AMOUNTS OF POTASSIUM (205 VS. 4 MG. PER LITER OF SOLUTION CULTURE) IN NUTRIENT SOLUTIONS SUPPLIED EITHER WITH NITRATE OR AMMONIUM SALTS ON THE AMOUNTS (MG. PER GM. OF FRESH TISSUE) OF ALPHA AMINO-, AMMONIUM- AND NITRATE-NITROGEN IN DIFFERENT SECTIONS OF THE LEAVES, STEM, AND ROOTS OF ONE-YEAR-OLD PLANTS OF *Ananas comosus* (L.) MERR.

PLANT SECTIONS	NITRATE-N						AMMONIUM-N					
	HIGH-K			LOW-K			HIGH-K			LOW-K		
	ALPHA-AMINO-N	NH_4-N	NO_3-N	ALPHA-AMINO-N	NH_4-N	NO_3-N	ALPHA-AMINO-N	NH_4-N	NO_3-N	ALPHA-AMINO-N	NH_4-N	NO_3-N
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Leaves												
Old (B) 1+2 (base)	0.302	0.009	0.294	0.400	0.009	0.060	0.313	0.016	0	0.610	0.007	0
3	0.289	0.007	0.080	0.400	0.009	0	0.291	0.011	0	1.000	0.007	0
4	0.357	0.008	0.018	0.406	0.010	0	0.350	0.013	0	0.730	0.009	0
5 (tip)	0.357	0.010	0	0.392	0.009	0	0.400	0.017	0	1.074	0.013	0
Mean	0.326	0.009	.....	0.400	0.009	.....	0.339	0.014	.....	0.854	0.009	.....
Mature (C) 1 (base)	0.234	0.007	0.285	0.312	0.008	0.133	0.255	0.013	0	0.463	0.013	0
2	0.253	0.009	0.270	0.332	0.009	0.027	0.204	0.012	0	0.708	0.010	0
3	0.297	0.009	0.083	0.447	0.010	0	0.330	0.011	0	0.913	0.008	0
4	0.385	0.009	0.010	0.497	0.010	0	0.378	0.012	0	1.377	0.007	0
5 (tip)	0.344	0.009	0	0.546	0.010	0	0.573	0.013	0	1.636	0.009	0
Mean	0.303	0.009	.....	0.427	0.009	.....	0.348	0.012	.....	1.019	0.009	.....
Active (D) 1 (base)	0.275	0.008	0.285	0.365	0.009	0.157	0.319	0.013	0	0.666	0.011	0
2	0.201	0.009	0.263	0.238	0.009	0.125	0.280	0.011	0	0.494	0.012	0
3	0.297	0.009	0.064	0.365	0.009	0	0.314	0.011	0	0.765	0.009	0
4	0.338	0.009	0	0.447	0.010	0	0.436	0.019	0	0.998	0.007	0
5 (tip)	0.357	0.011	0	0.682	0.014	0	0.560	0.017	0	1.277	0.009	0
Mean	0.294	0.009	.....	0.419	0.010	.....	0.382	0.014	.....	0.840	0.010	.....
Young (E) 1 (base)	0.398	0.012	0.158	0.403	0.016	0.143	0.288	0.018	0	0.627	0.008	0
2	0.302	0.012	0.058	0.282	0.009	0.047	0.302	0.014	0	0.522	0.011	0
3	0.322	0.009	0.016	0.326	0.010	0	0.248	0.012	0	0.472	0.013	0
4+5 (tip)	0.352	0.010	0	0.542	0.010	0	0.392	0.012	0	1.000	0.005	0
Mean	0.344	0.011	.....	0.388	0.011	.....	0.307	0.014	.....	0.655	0.009	.....
Leaves, mean	0.315	0.010	.....	0.410	0.010	.....	0.346	0.014	.....	0.850	0.009	.....
Stem												
Base	0.341	0.016	0.135	0.373	0.013	0.054	0.392	0.015	0	0.544	0.007	0
Middle	0.357	0.012	0.193	0.456	0.014	0.054	0.386	0.013	0	0.766	0.011	0
Apex	0.426	0.010	0.385	0.538	0.017	0.068	0.487	0.016	0	1.000	0.017	0
Mean	0.375	0.013	.....	0.456	0.015	.....	0.422	0.015	.....	0.770	0.012	.....
Roots	0.192	0.009	0.086	0.332	0.010	0.100	0.294	0.012	0	0.301	0.005	0

amounts of the various inorganic and organic nitrogenous fractions in *Ananas comosus*. The higher concentrations of potassium increased the nitrate content of the tissues in the N-n series, and, in consequence, the values of total-N, soluble organic-N, and protein-N per plant, reported in table II, were greater in the High-K than in the Low-K cultures. The nitrogen values in table I, reported as milligrams per gram of tissue, are different from those in table II which are presented on a per plant or organ basis. However, the results obtained by either method yield information differentiating the total (table II) from the relative (table I) amounts of the various nitrogenous fractions in the plant.

The rate of nitrogen absorption by roots from nutrient solutions, indicated by the total nitrogen content per plant in table II (sum of ammonium, nitrate, soluble organic-N, and protein-N) for the same growth period, varied in the different cultures. The difference in total nitrogen values between High-K and Low-K cultures in the N-n series was 16.15 per cent. higher for the former cultures; whereas a similar difference in the A-n series, also in favor of the High-K cultures, was 2.6 per cent. The difference in series being considerably smaller in the A-n than N-n series, suggests that the rate of nitrogen absorption by the roots of either the High-K or Low-K cultures was approximately the same or slightly in favor of the former cultures, in spite of the larger root system of the Low-K cultures in the A-n series. But in the N-n series a difference many times greater than in the A-n series indicates that the rate of nitrate absorption by the High-K cultures was higher than by the Low-K cultures. Therefore, the data may indicate that absorption of ammonium nitrogen was not inhibited by either concentration of K ions in the A-n series, but in the N-n series the higher K-ion concentration had increased nitrate absorption.

Protein-N as percentage of total organic-N in the N-n series was 57.1 and 53.7 per cent. for the High-K and Low-K cultures, respectively. Similar values in the A-n series were 56.7 and 42.3 per cent. for the High-K and Low-K cultures, respectively. The difference between High-K and Low-K cultures being smaller in the N-n than A-n series (3.4 vs. 14.4) might indicate in the former series that the rate of nitrate absorption by roots, its assimilation to soluble organic-N, and conversion of the latter to protein-N were coordinated by the supply of K ions. But in the A-n series coordination was lacking between the supply of K ions and ammonium absorption by roots and polymerization of the primary products of assimilation. The apparently regulatory effects of K ions on nitrate absorption by roots retarded the accumulation of much greater amounts of soluble organic-N in Low-K cultures in the N-n series supplied with nitrate, and thus a satisfactory ratio was maintained between soluble organic-N and protein-N. Also, the possible accumulation in plant tissues of NO_3 ions from external sources but not of NH_4 ions, except in small amounts, in cultures supplied with nitrate and ammonium, respectively, may influence greatly, by their relative degree of assimilability, the ratio of soluble organic-N to protein-N. There-

fore, the relatively great accumulations of soluble organic-N in both High-K and Low-K cultures in the A-n series should be attributed to the inability of K ions to regulate the absorption of NH_4 ions by roots. Also, the much greater amounts of soluble organic-N than protein-N in the Low-K than High-K cultures in the A-n series should be attributed to the inadequacy of the low concentrations of K ions to promote conversion of soluble organic-N to protein-N. Doubtless, all reactions involved in the conversion of soluble organic-N to protein-N are of enzymatic nature and may require as substrata for protein synthesis other substances besides the various fractions in the soluble organic-N complex. Therefore, different K-ion concentrations may modify in some obscure manner the rate of enzymatic activity in the conversion of soluble organic-N to protein-N. Such interpretations of the physiological action of potassium are plausible because they are supported in part by the experimental data reported above.

The theories of various investigators on the effects of K ions in protein synthesis are manifold and divergent. STEWART, STOUTE, and PRESTON (**25, 26**) claim that the stable amide and amino acid fractions in dormant potato tubers were found to be sources of nitrogen for protein formation and that potassium salts appeared to increase and calcium salts to decrease their relative utilization. WALL (**29, 30, 31**) observed that in K-deficient cultures tomato plants made better growth when supplied with nitrate than with ammonium; the latter, showing rapid breakdown of leaf tissues due to high internal ammonium-ion concentration, increases in amide and amino acid nitrogen and decreases in protein-nitrogen. GREGORY (**2**), quoting the results of SAÏD (**15**), SANKARAN (**16**), and SCHWABE (**18**) which indicate that the protein content of potassium-deficient leaves was high at first but decreased later, states that protein synthesis as well as hydrolysis must be taking place very rapidly and that the effect of potassium deficiency is related to amino nitrogen content because the level of this fraction determines the rate of proteolysis. This interpretation of GREGORY, although suggesting an additional explanation for the accumulations of amino nitrogen in the Low-K cultures, does not conform in all respects with our experimental evidence, indicating that the protein content of the leaves of the Low-K cultures, in table I, was as high or higher than that of the High-K cultures, suggesting either very little or no proteolysis in the former cultures.

The data in tables I and II reveal that the protein content of the stem was consistently higher in the High-K than in the Low-K cultures. Approximately similar results were obtained by other investigators. WALL's results (**29**) show that protein nitrogen as percentage of total nitrogen in the upper and lower stem sections of tomato plants grown in cultures with 351, 176, 44 or 0 p.p.m. of K were, respectively, 76.0, 60.0, 69.0 and 51.0 per cent. for the upper stem and 56.0, 55.0, 47.0 and 45.0 per cent. for the lower stem, showing a higher protein content in both stem sections of the plus potassium than minus potassium cultures. BURRELL's (**1**) findings in soybean plants showed percentage protein values on the fresh weight basis in the stem as 0.233 and

0.167 and in the leaves as 0.125 and 0.147 for the plus-K and minus-K cultures, respectively. HARTT (6) has obtained results indicating that in *Saccharum officinarum* L. protein-nitrogen values of the stem were higher for the plus-K than minus-K cultures and that amino-nitrogen values of the leaves were higher in the minus-K than in the plus-K cultures. The data of NIGHTINGALE *et al.* (8) show in many cases good agreement with the results of this study, but the differences in stem protein-nitrogen between plus-K and minus-K cultures are small. The findings of PHILLIPS, SMITH, and HELPER (11) on the protein-nitrogen content of the stem of tomato plants are partly in disagreement with the data in this study as these investigators claim, "that a moderate but definite degree of potassium deficiency had been reached in the plants not supplied with potassium." Their data show that the K content of the upper-stem tissues of the K-deficient plants was from 50 to 75 per cent. as high as that of the plus-K cultures. In WALL's studies (29) where high and low protein-N in the upper stem of the tomato correlated with the plus-K and minus-K cultures, respectively, the potassium content of the upper stem in the minus-K plants was approximately 34.0 per cent. as high as that of the plus-K cultures. The difference between minus-K and High-K cultures of 50 or 75 per cent., in the study of PHILLIPS *et al.* (11), and 34 per cent., in that of WALL (29), in the potassium content of the tissues, being sufficiently great, might explain the negative results of PHILLIPS.

Unpublished data from experiments with *A. comosus* grown under field conditions and supplied with different amounts of potassium have not fully confirmed the findings reported in this study with respect to the relative amounts of starch or protein in High-K or Low-K cultures. However, the failure of the field-grown plants to yield data comparable to those obtained in nutrient solutions should be attributed to the relatively high initial potassium content of the soil which was many times higher than that supplied to the nutrient solutions of the Low-K cultures. The findings suggest that unless the initial potassium supply in the soil is very low the starch and protein-N content of the tissues of Low-K cultures may be as high or higher than that of High-K cultures on account of restricted growth activity and a lower rate of utilization of the products of photosynthesis and assimilation by the former than the latter cultures.

The above comparisons show that differences in leaf protein-nitrogen between plus-K and minus-K cultures, although in some cases in favor of the former and in others of the latter cultures, fail to correlate in all cases with the amounts of potassium supplied to the roots. However, they show that in the stem and especially in the meristematic tissues of this organ, protein-N was higher in the plus-K than in the minus-K cultures. The meristematic tissues of the peripheral and apical regions of the stem, associated with practically all growth activities in *A. comosus*, contained higher values of protein-N, presumably of protoplasmic origin, in the High-K than Low-K cultures, indicating a positive correlation between high amounts of potassium, protein-N, and plant growth. However, in the highly differenti-

ated tissues of fully expanded leaves a similar correlation was lacking, or a negative correlation could be observed in most sections. STEWART and PRESTON's results (**26**) with potato discs have indicated that potassium salts stimulate respiration and protein synthesis from stored amino acids while calcium salts depress both processes; also, that amino acids do not yield protein directly but are first deaminated by oxidizing enzymes releasing ammonia and probably a keto acid which contributes to the substrates of aerobic respiration. In a previous study (**21**), the authors suggested that potassium may also promote the conversion of reducing sugars to sucrose or starch. The details in the physiological function of potassium for promoting the conversion of soluble organic nitrogen fractions to protein nitrogen are not known. However, the manifold activity of potassium suggests that its effects are directly concerned with the activity of the protoplasm which may increase or decrease, presumably by enzymatic action, the rate of sucrose, starch, or protein-N polymerization in certain parenchymatous or meristematic tissues, depending on the physiological status of the plant under the influence of different K-ion, NO_3-ion, or NH_4-ion concentrations.

Soluble organic-N accumulations in the leaves of the Low-K cultures, attributable by GREGORY (**2**) to an increased rate of proteolysis, might have resulted from a lower rate of utilization in protoplasm-forming substances on account of much smaller plant weights in the Low-K than High-K cultures. Previous studies (**25**) have shown that similar accumulations may be found in apparently healthy tissues of plants exposed to adverse conditions. Such accumulations, resulting from translocations of soluble organic-N from injured or dead tissues where it was generated by enzymic hydrolysis of proteins, should not be confused with those in plants lacking symptoms of visual injury or death of tissues. In K-deficient cultures of *A. comosus*, having developed serious injury of the terminal regions of the leaves known as leaf tip necrosis, accumulations of soluble organic-N may result from hydrolysis of the proteins of the dead or injured tissues. However, as differentiation of soluble organic-N fractions, resulting either from hydrolysis of dead tissue proteins or from synthesis by inorganic-N assimilation, is impossible, the accumulations of soluble organic nitrogen in Low-K cultures cannot be assigned definitely to a single source.

Summary

1. High-potassium cultures (205 mg. per l.) compared with low-potassium cultures (4 mg. per l.), supplied with equal amounts of nitrogen either as nitrate or ammonium, modified the relative amounts of different nitrogenous fractions in the tissues of *A. comosus*.

2. Nitrate nitrogen, as indicated by the nitrate content of the basal leaf and stem sections, was greater in the high-potassium than in the low-potassium cultures in the nitrate series. Ammonium nitrogen, generated possibly by deaminizing processes, occurred in traces in the plants of both nitrate and ammonium series. Ammonium absorbed from nutrient solutions was readily

converted to compounds of greater complexity in the roots, and it is doubtful that any passed from roots to stem unchanged.

3. Soluble organic nitrogen calculated as mg. per gram of fresh weight was higher in the low-potassium than high-potassium cultures of the nitrate series. When calculated as total soluble organic nitrogen per plant, the values were reversed, being greater in the high-potassium than low-potassium cultures. In the ammonium series the values of soluble organic nitrogen, calculated by either method, were greater in low-potassium cultures. Alpha amino-N, a fraction of soluble organic-N, was higher in the low-potassium than in the high-potassium cultures of both series, but in the ammonium series the values were considerably higher than in the nitrate series.

4. Protein nitrogen in the leaves, calculated as mg. per gram of tissue, was greater in the low-potassium than in the high-potassium cultures, but in the stem these values were reversed, being greater in the high-potassium than in the low-potassium cultures. Calculated as total protein nitrogen per organ or plant, the values were greater for the high-potassium than low-potassium cultures in both leaves and stem.

5. The data indicate that the physiological function of potassium in the conversion of soluble organic nitrogen fractions to protein nitrogen in high-potassium cultures is more noticeable in the stem than in the highly differentiated tissues of fully expanded leaves. In the leaves protein-N was higher in the low-potassium than high-potassium cultures. The higher content of protein-N in the stems of the high-potassium than low-potassium cultures is attributed to the conditions in the peripheral and apical regions of the stem which, composed mostly of meristematic tissues and possessing potential growth activity, require greater quantities of proteins for the regeneration of protoplasm than similar tissues of the low-potassium cultures which lack equally great growth potentialities.

PINEAPPLE RESEARCH INSTITUTE
UNIVERSITY OF HAWAII
HONOLULU, T. H.

LITERATURE CITED

1. BURRELL, R. C. Effect of certain deficiencies on nitrogen metabolism of plants. Bot. Gaz. **82**: 320–328. 1926.
2. GREGORY, F. G. Mineral nutrition of plants. Ann. Rev. Biochem. **6**: 557–578. 1937.
3. ———, and BAPTISTE, E. C. D. Physiological studies in plant nutrition. V. Carbohydrate metabolism in relation to nutrient deficiency and to age in leaves of barley. Ann. Bot. **50**: 579–619. 1936.
4. ———, and RICHARDS, F. J. Physiological studies in plant nutrition. I. The effect of manurial deficiency on the respiration and assimilation rate in barley. Ann. Bot. **43**: 119–161. 1929.
5. ———, and SEN, P. K. Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitro-

gen metabolism of the barley leaf as determined by nitrogen and potassium deficiency. Ann. Bot. n.s. **1**: 521–561. 1937.

6. HARTT, C. E. Some effects of potassium upon the amounts of protein and amino forms of nitrogen, sugars, and enzyme activity of sugar cane. Plant Physiol. **9**: 453–490. 1934.
7. HIBBARD, R. P., and GRIGSBY, B. H. Relation of light, potassium, and calcium deficiencies to photosynthesis, protein synthesis, and translocation. Michigan Agr. Expt. Sta. Tech. Bull. **141**. 1934.
8. NIGHTINGALE, G. T., SCHERMERHORN, L. G., and ROBBINS, W. R. Some effects of potassium deficiency on the histological structure and nitrogenous and carbohydrate constituents of plants. New Jersey Agr. Expt. Sta. Bull. **499**. 1930.
9. PHILLIPS, T. G., and SMITH, T. O. The effect of potassium deficiency on metabolism in plants. New Hampshire Agr. Expt. Sta. Bull. **26**. 1928.
10. ————, ————, and DEARBORN, R. B. The effect of potassium deficiency on the composition of the tomato plant. New Hampshire Agr. Expt. Sta. Tech. Bull. **59**. 1934.
11. ————, ————, and HELPER, J. R. Some effects of potassium and nitrogen on the composition of the tomato plant. New Hampshire Agr. Expt. Sta. Tech. Bull. **73**. 1939.
12. RICHARDS, F. J. Physiological studies in plant nutrition. III. Further studies on the effect of potash deficiency on the rate of respiration in leaves of barley. Ann. Bot. **46**: 367–388. 1932.
13. ————, and TEMPLEMAN, W. G. Physiological studies in plant nutrition. IV. Nitrogen metabolism in relation to nutrient deficiency and age in leaves of barley. Ann. Bot. **50**: 367–402. 1936.
14. ROHDE, G. Die Wirkung des Kaliums bei der Kohlensäure-assimilation der Pflanzen. Zeitschr. Pflanzenernähr. Düngung und Bodenk. **44**: 1–24. 1936.
15. SAÏD, H. Thesis. Imp. Coll. Sci., London. 1934.
16. SANKARAN, R. Thesis. Univ. of London. 1936.
17. SCHMALFUSS, KARL. Untersuchungen über den Eiweissstoffwechsel von Kalimangelpflanzen. Ein Beitrag zur Kenntnis der physiologischen Rolle des Kaliums in der Pflanze. Phytopath. Zeitschr. **5**: 207–249. 1933.
18. SCHWABE, G. Über die Wirkung der Amino-säuren auf den Sauerstoffverbrauch submerser Gewächse. Protoplasma **16**: 397–451. 1932.
19. SIDERIS, C. P., and YOUNG, H. Y. Effects of iron on certain nitrogenous fractions of *Ananas comosus* (L.) Merr. Plant Physiol. **21**: 75–94. 1946.
20. ————, and ————. Effects of potassium on growth and ash constituents of *Ananas comosus* (L.) Merr. Plant Physiol. **20**: 609–630. 1945.
21. ————, and ————. Effects of potassium on chlorophyl-

lous pigments, ascorbic acid, acidity and carbohydrates of *Ananas comosus* (L.) Merr. Plant Physiol. **20**: 649–670. 1945.

22. ————, Krauss, B. H., and Young, H. Y. Assimilation of ammonium and nitrate by pineapple plants grown in nutrient solutions and its effects on nitrogenous and carbohydrate constituents. Plant Physiol. **13**: 489–527. 1938.
23. ————, ————, and ————. Distribution of different nitrogen fractions, sugars, and other substances in various sections of the pineapple plant grown in soil cultures and receiving either ammonium or nitrate salts. Plant Physiol. **14**: 227–254. 1939.
24. ————, ————, and ————. Distribution of nitrogenous fractions, sugars, and other substances in *Ananas* grown in darkness vs. daylight. Plant Physiol. **14**: 647–676. 1939.
25. Stewart, F. C., and Preston, G. Metabolic processes of potato discs under conditions conducive to salt accumulation. Plant Physiol. **15**: 23–62. 1940.
26. ————, and ————. The effect of salt concentration upon the metabolism of potato discs and the contrasted effect of potassium and calcium salts which have a common ion. Plant Physiol. **16**: 85–116. 1941.
27. ————, Stoute, P. R., and Preston, C. The balance sheet of metabolites for potato discs showing the effect of salts and dissolved oxygen on metabolism at 23 degrees C. Plant Physiol. **15**: 409–448. 1940.
28. Van Slyke, D. D. A method for quantitative determination of aliphatic groups. Jour. Biol. Chem. **9**: 185–204. 1911.
29. Von Turtschin, Th. W. Einfluss des Kalis auf den Stickstoff und Kohlenhydratenwechsel in Pflanzen. Zeitschr. für Pflanzenernährung A. XXXIV. 1934.
30. Wall, M. E. The rôle of potassium in plants. I. Effect of varying amounts of potassium on nitrogenous, carbohydrate, and mineral metabolism in the tomato plant. Soil Sci. **47**: 143–161. 1939.
31. ————. The rôle of potassium in plants. II. Effect of varying amounts of potassium on the growth status and metabolism of tomato plants. Soil Sci. **49**: 315–331. 1940.
32. ————. The rôle of potassium in plants. III. Nitrogen and carbohydrate metabolism in potassium-deficient plants supplied with either nitrate or ammonium nitrogen. Soil Sci. **49**: 393–410. 1940.

PHYCOMYCES IN THE ASSAY OF THIAMINE IN AGAR

DOROTHY DAY AND ANNETTE H. HERVEY[1]

Introduction

In the course of experiments conducted by DAY (1), it was observed that an agar culture with a known quantity of thiamine produced a lesser dry weight of *Phycomyces Blakesleeanus* than was to be expected from a previously determined dry weight of the organism grown in liquid culture with the same quantity of thiamine. The purpose of this investigation was to find out whether or not the use of heat in removing and washing the mycelium grown on the agar media was responsible for this difference in dry weight. The inhibiting effect of agar on the growth of Phycomyces also was investigated.

Materials and methods

Phycomyces Blakesleeanus was grown at 25° C. under the conditions used by DAY (1). The basal solution contained per liter of distilled water: 1.5 gm. KH_2PO_4, 0.5 gm. $MgSO_4 \cdot 7H_2O$, 50.0 gm. dextrose, 2.0 gm. asparagine, and mineral supplements in p.p.m. as follows: B, 0.005; Cu, 0.02; Fe, 0.10; Mn, 0.01; Mo, 0.01; and Zn, 0.09. Purified Bacto agar and thiamine were added to this basal solution in various proportions and combinations. In each experiment, the mycelium was dried for 24 hours at 100° C. after its removal from the medium by two or more of the following methods with four or eight flasks for each method. 1) Cultures to which hot, distilled water was added were heated until the agar melted. The mycelial mats taken from four similar flasks were washed in two successive beakers of hot (95°—100° C.) distilled water before being placed in an aluminum pan to dry. 2) The flasks were not heated, and cold, distilled water (room temperature) was used throughout. 3) After the addition of cold water to the cultures, the entire contents of four similar flasks were emptied into a Gooch crucible containing a piece of fine cheesecloth as a filter, and one liter of cold water was flooded through the mycelium. 4) A similar crucible with suction was employed, but hot water was used throughout.

Results

DRY WEIGHT OF MYCELIUM GROWN IN AGAR MEDIA

The mycelium of Phycomyces grown in agar, taken from a flask, and washed in cold water felt slimy before drying; with 1.0 per cent. agar, obvious lumps still adhered to the mycelium. It was impossible to remove all mycelium from the semi-solid agar, as was shown by bits of mycelium floating in the flask when the contents were heated after the mycelium had supposedly been removed. The mycelium filtered and washed with cold

[1] The experimental part of this research was completed at Smith College while both authors were members of the Department of Botany.

water as described in 3 above felt slimy but was less so than that treated by method 2, indicating more successful separation of mycelium from the medium. With hot water, the mycelium did not feel slimy, and the agar appeared to be completely removed by washing in beaker or crucible.

The dry weight of the mycelium grown in agar and washed with cold water was greater than the comparable dry weight of similar mycelium washed with hot water; this relationship was consistent for both pans and crucibles in every experiment regardless of the amount of thiamine added to the culture (table I). The difference was greatest for pan-dried mycelium

TABLE I

DRY WEIGHTS OF MYCELIUM OF *Phycomyces Blakesleeanus* WASHED WITH HOT OR COLD WATER AND DRIED IN PANS OR CRUCIBLES

	WASHED IN COLD WATER			WASHED IN HOT WATER			
THIAMINE IN mμ MOLES	0.1	0.2	0.5	0.1	0.2	0.5	1.0
PERCENTAGE AGAR	MYCELIUM TAKEN FROM FLASK AND WASHED IN PANS*						
0.0 (liquid)	16.1	33.8	70.9	14.5	26.9	55.9	96.4
0.5	17.0	31.0	78.3	12.6	24.3	58.8	103.2
1.0	32.2	42.0	84.1	12.4	24.5	56.5	110.3
2.0	.....	.....	.....	11.8	21.6	53.0	108.2
3.0	.....	.....	.....	10.7	18.2	49.6	99.9
4.0	.....	.....	.....	10.5	15.1	45.4	94.2
	MYCELIUM FILTERED AND DRIED IN CRUCIBLES*						
0.0 (liquid)	18.2	30.7	65.2	15.3	29.4	57.2	.....
1.0	20.0	38.9	82.7	14.2	26.3	54.3	.....

* Dry weight is recorded in mg. per culture; each figure represents the average of from 8 to 32 cultures.

from 1.0 per cent. agar, where the mycelium washed in cold water was 49 to 159 per cent. heavier than comparable mycelium washed in hot water (table I). Therefore, it appeared that mycelium grown on agar and washed with cold water had a higher dry weight than did that washed with hot water, apparently because the unremoved agar in addition to the mycelium gave a higher dry weight than the mycelium alone would have given.

DRY WEIGHT OF MYCELIUM GROWN IN LIQUID MEDIA

The dry weight of the mycelium washed in cold water was 4 to 27 per cent. greater than similar mycelium washed with hot water (table I). Thus it appeared that washing in hot water lowered the resulting dry weight of the mycelium, probably by dissolving soluble substances out of it. The same effect may be assumed when hot water was used to wash similar mycelium grown in agar.

Effect of concentration of agar on Phycomyces

Dry weights of mycelium grown with 0.1 mμ mole and with 0.2 mμ mole thiamine in two series of cultures containing from 0.0 to 1.0 per cent. agar by 0.2 per cent. steps showed that these amounts of agar had no effect on the growth of the organism. In subsequent experiments with thiamine ranging from 0.1 mμ mole to 1.0 mμ mole, the dry weight of mycelium grown in the liquid medium was from 13 per cent. less to 17 per cent. more than the dry weight of mycelium grown with 1.0 per cent. agar (table I). Tests with larger proportions of agar and various amounts of thiamine did not show a perfectly consistent relationship between the amount of agar and the resulting dry weight of the mycelium, but more agar was generally accompanied by less growth of mycelium.

Discussion

It is clear from these results that the use of cold water in washing Phycomyces mycelium which had grown in an agar medium did not result in the complete removal of the agar. Sufficient agar remained to affect materially the dry weight of the mycelium. Although the use of hot water in washing the mycelium grown in the agar medium resulted in the successful removal of the agar, enough soluble material was leached from the hyphae during the washings to influence the dry weight. While these results were to be expected, it was not possible to estimate the amount of the effects without the actual determinations. The differences found are sufficiently great to be significant if Phycomyces is used for the quantitative determination of thiamine.

The reduction in growth demonstrated with the higher concentrations of agar may have been caused by the physical resistance of the agar to the growth of the organism, the lessened availability of water, thiamine, or other components of the medium, or the presence of some unknown toxic factor in agar.

Summary

Phycomyces Blakesleeanus was grown in a basal medium of distilled water, minerals, dextrose, and asparagine plus purified agar and thiamine in various proportions and combinations. The mycelium was harvested by the use of hot and cold water in methods which included the use of aluminum pans and Gooch crucibles.

With the basal liquid medium the dry weight of mycelium washed with cold water was higher than that washed with hot water, probably because of the action of hot water on the mycelium in dissolving substances from it.

The higher dry weight of mycelium grown on agar and washed with cold water as contrasted with that washed with hot water might be explained not only by this action of hot water but also by the incomplete separation of the agar and the mycelium with cold water.

Reduced growth of Phycomyces was generally correlated with an increased amount of agar.

Grateful appreciation is expressed to W. J. Robbins for critical reading of the manuscript.

Division of Plant Pathology and Agricultural Botany,
University Farm,
St. Paul, Minnesota, and
Columbia University, New York

LITERATURE CITED

1. Day, Dorothy. Thiamin content of agar. Bull. Torrey Club **69**: 11–20. 1942.

LEAFSPOT OF PEANUT ASSOCIATED WITH MAGNESIUM DEFICIENCY

ROGER W. BLEDSOE, HENRY C. HARRIS, AND W. B. TISDALE

(WITH TWO FIGURES)

It is generally recognized that the susceptibility and prevalence of many plant diseases are associated with plant nutrition. The peanut plant is susceptible to leafspot infection which becomes economically important during the last half of its growth period when the crop is planted in the spring. The severity and rapid spread of infection in the field may not be due to the mere presence of the organism, which is ubiquitous, but to the susceptibility of plants suffering from improper nutrition in certain stages of their growth. Results herein reported of a nutritional study with the Dixie Runner variety of peanut support this assumption.

The experimental peanut plants were grown in sand culture with the roots of each plant in a single gallon glass jug which completely isolated the roots from the fruiting or pegging zone of the plant (fig. 1). The nutrients were applied to the rooting medium by the continuous flow method of SHIVE and STAHL (**6**), while the nutrients on the pegging zone were applied on the surface and by feeding through the base of the box. For 75 days after germination the rooting and fruiting zones of all cultures were supplied HOAGLAND'S (**4**) no. 1 complete nutrient solution modified by reducing the concentration of the macronutrients by half and the micronutrients to one-tenth. Ferrous iron was added as needed. At the end of that period the following nutrient treatments were begun on the rooting medium: complete, minus phosphorus, minus potassium, minus calcium, minus magnesium, minus sulphur, and minus micronutrient elements. The complete nutrient solution was applied to the fruiting medium throughout the experiment.

There were three replicates of single plants to each treatment and the roots of each culture received 1.5 liters of nutrient solution daily; the pegging region received 3.5 liters of solution weekly. Plants were grown 21 days in the greenhouse after germination and then because of high temperature were moved outside under a frame covered with one thickness of cheesecloth. This report will deal only with the results obtained with the magnesium-deficient cultures, since these plants were the only ones that developed the leafspot disease to any appreciable extent under the conditions of this experiment.

The leaves of the minus-magnesium plants showed interveinal chlorotic centers with green margins 24 days after magnesium was withheld from the roots. However, the effect appeared initially on the first leaf below the growing tip of the oldest branches. This is in contrast to the typical magnesium deficiency on other plants which usually occurs first on the basal

leaves. At the end of 35 days the deficiency symptom was apparent on the young leaves of all branches including the main shoot and had progressed four to five leaves below the growing tips of the oldest branches. The chlorosis of the affected leaves became more severe with advancing age.

On July 28, when plants were 103 days old, and magnesium had been withheld for 28 days, leafspot was observed on those leaves which first showed symptoms of the nutritional disturbance. A fungus (*Mycosphaerella arachidicola* W. A. Jenk.) was isolated from the spots. The progress of the disease followed the same order of development as that of the deficiency symptom, affecting first the young leaves of the oldest branches and then

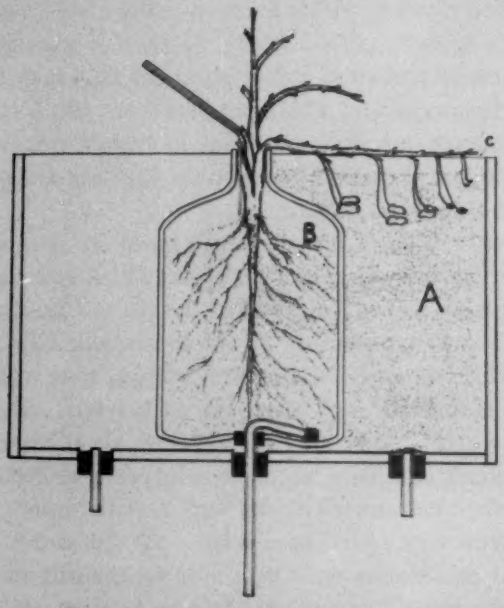


FIG. 1. Diagrammatic view of peanut plant showing: A, fruiting medium; B, rooting medium; and C, string mesh to prevent contact and rooting of branches in the sand.

proceeding to the young growth of all branches. Leafspot on plants in the field usually attacks the basal leaves first and advances progressively toward the tips of branches.

Leafspot counts were made to determine the incidence of disease on the plants of the minus-magnesium and complete-nutrient solutions on August 10, when plants were 116 days old and magnesium had been withheld for 41 days (table I). The counts were made on the terminal and basal leaves of the same branch, and the disease was highly correlated with the area of the deficiency symptom (fig. 2).

Magnesium determination by the LINDNER (5) method was made of basal, central, and terminal leaves from branches of the same physiological age (table II). The leaf material for analysis was collected at the time the leafspots were counted.

The chemical analyses showed a decrease in magnesium concentration

TABLE I

INCIDENCE OF LEAFSPOT ON PEANUT PLANTS GROWN IN COMPLETE NUTRIENT SOLUTION AND A SOLUTION WITH MAGNESIUM WITHHELD FOR 41 DAYS (AGE OF PLANTS, 116 DAYS)

TREATMENT	NO. OF PLANTS	NO. OF BRANCHES	NO. OF LEAFLETS	NUMBER OF LEAFSPOTS	
				TERMINAL LEAFLETS	BASAL LEAFLETS
Complete nutrient solution	3	15	180	6	15
Minus magnesium	3	15	204	3502	30

from the basal to the terminal leaves, and spectrographic analyses substantiated those results.

No precaution was taken to prevent spread of the leafspot. Moreover, daily flower counts were made on all cultures when the foliage was wet from dew or rain and close spacing of plants caused branches from other cultures to overlap those of the magnesium-deficient plants. The prevalence of leafspot on plants grown on a deficiency of the elements other than magnesium was no greater than that of the controls even though the given deficiency was severe, and the plants were much weakened.

The plants which had magnesium withheld from the roots had many fruit developing in the fruiting medium which contained magnesium. BURKHART and COLLINS (2) have shown that the growing peanut fruit is capable of absorbing minerals. They further state that gypsum applications condition the peanut plant in such a manner that the foliage becomes very susceptible to leafspot injury.

CHAPMAN (3) states that prolonged magnesium deficiency of citrus

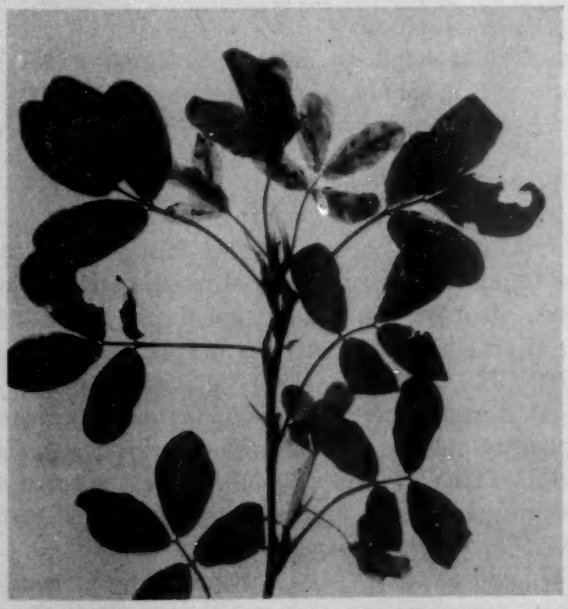


FIG. 2. Branch from peanut plant showing prevalence of leafspot on chlorotic leaflets on August 10, after withholding magnesium from the rooting medium for 41 days.

TABLE II

MAGNESIUM IN PER CENT. OF DRY WEIGHT OF PEANUT LEAVES FROM DIFFERENT POSITIONS ON PLANTS GROWN IN MAGNESIUM-DEFICIENT AND COMPLETE-NUTRIENT SOLUTIONS

POSITION OF LEAVES ON BRANCH	MINUS MAGNESIUM	COMPLETE
	%	%
Terminal	0.012	0.307
Central	0.022	0.317
Basal	0.082	0.401

grown in solution culture brings on typical iron chlorosis in the leaves. BENNETT (1) suggests that the availability of iron might be influenced by other factors. Liberal amounts of ferrous iron were supplied to the rooting and fruiting mediums of the minus magnesium peanut plants in this experiment. Furthermore, there was no response to iron or other micronutrients when solutions were streaked or spread on the surface of the chlorotic leaflets. While the nutrient disturbance was associated with magnesium deficiency, it is entirely possible that it was caused by an interaction of ions.

Visual symptoms of magnesium deficiency of field-grown peanuts have not been observed. However, that does not preclude the possibility that leafspot is induced by a deranged metabolism resulting from magnesium deficiency. In this study it appears that the low level of magnesium was either directly or indirectly responsible for the susceptibility of peanut to leafspot. The exact nature of the nutrient disturbance, as well as whether or not the severity of leafspot can be reduced by higher levels of magnesium in the plant, must await further experimentation.

The authors wish to thank MR. THOMAS C. ERWIN of the Department of Soils for the spectrographic analyses.

FLORIDA AGRICULTURAL EXPERIMENT STATION
GAINESVILLE, FLORIDA

LITERATURE CITED

1. BENNETT, J. P. Iron in leaves. Soil Sci. **60**: 91–105. 1945.
2. BURKHART, L., and COLLINS, E. R. Mineral nutrients in peanut plant growth. Soil Sci. Soc. Amer. Proc. **6**: 272–280. 1941.
3. CHAPMAN, H. D. Mineral nutrition of plants. Ann. Rev. Biochem. **14**: 709–732. 1945.
4. HOAGLAND, D. R., and ARNON, D. I. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Cir. **347**. 1938.
5. LINDNER, R. C. Rapid analytical methods for some of the more common inorganic constituents of plant tissues. Plant Physiol. **19**: 76–89. 1944.
6. SHIVE, J. W., and STAHL, A. L. Constant rates of continuous solution renewal for plants in water cultures. Bot. Gaz. **84**: 317–323. 1927.

NOTES

The St. Louis Meeting.—The American Society of Plant Physiologists held its twentieth annual meeting at Saint Louis, Missouri, on March 28–30. The Statler Hotel was the official headquarters and the majority of the meetings were held there. Most of the papers were presented in a series of two parallel sessions jointly held with the Physiological Section of the Botanical Society of America. In addition, a joint session was held with Section G, Botanical Society of America, American Phytopathological Society, Mycological Society of America, and the Sullivant Moss Society on Thursday afternoon and a second joint session was held with Section O, the American Society for Horticultural Science on Friday afternoon. Both sections of the physiological program were well attended.

The banquet was held in the Mark Twain Hotel on Friday evening, with President Paul J. Kramer presiding. Despite conflicts with dinners of other societies, the number attending the dinner for plant physiologists was greater than in pre-war years. Since no meeting had been held in 1945, two presidential addresses were given. Dr. B. S. Meyer, president of the society during 1943–44, presented a paper entitled "The Case for Greater Cooperation Among Plant Science Societies". Dr. H. A. Spoehr, the retiring president for 1944–45, spoke on "The Coming of Age of the American Society of Plant Physiologists". The Charles Reid Barnes Life Membership Award was made to Dr. D. R. Hoagland.

The Executive Committee held two meetings. The committee authorized publication of a new directory of members in the fall of 1946. The president was empowered to appoint two new committees, one on industrial relations and the second on the professional status of plant physiologists. Dr. R. Bouillenne of the Liege Botanical Institute, Belgium, was elected as a corresponding member. Storage of the archives of the Society at Miami University, Oxford, Ohio, was authorized. The Executive Committee approved action by the Society on the following constitutional changes, namely: that Article VIII, paragraph 2, sentence 2 be changed to read: "In case of a tie for fourth place, the secretary shall place the additional name or names on the ballot"; Article VIII, paragraph 4, be changed to read; "If two or more persons shall receive an equal number of votes for any office, the names of these persons shall receive an equal number of votes for any office, the names of these persons shall be resubmitted to the society"; Section 10 was amended to increase the Charles Reid Barnes Fund to $4000.

The annual business meeting was held on Thursday afternoon. After acceptance of the officers' and committees' reports, it was voted by the attending members that the editor-in-chief be authorized to publish dates of receipt and final acceptance of papers published in Plant Physiology.

The report of the Executive-Secretary Treasurer indicated that the Society is in a sound financial condition. With total endownments of $12,000, savings of $5000, and cash on hand to the amount of $8500, the Society should be able to carry on the usual activities even with rising costs

on all printing. The total membership is now 630 with subscriptions amounting to 575 in addition. An appeal is made for new members and subscriptions. The volume of printing reduces the cost of each succeeding copy, thus increasing the financial and professional strength of the society proportionately. Foreign memberships and subscriptions are rising slowly but steadily. Reserves of the journal make it possible to supply all missing back numbers.

The Charles Reid Barnes Award.—Professor Dennis Robert Hoagland of the University of California was awarded the Charles Reid Barnes Life Membership. Professor Hoagland was born in Golden, Colorado, on April 2, 1884. Upon graduation from Stanford University in 1907, he went to the University of California as instructor in Agricultural chemistry. Three years later he became chemist of the U. S. Department of Agriculture. After obtaining an advanced degree from the University of Wisconsin, he returned in 1913 to the University of California, where he has held professorships in agricultural chemistry and plant nutrition, and has been head of the division of plant nutrition since 1921.

Professor Hoagland soon became a leader in the field of the mineral nutrition of plants, and for the past thirty years his laboratory has continuously maintained its position among the foremost in the world. He has conducted a series of brilliant investigations on the nature of the soil solution, climatic influences on salt absorption, the accumulation of salts by plant cells, the dependence of accumulation upon metabolism, the role of the microtrophic elements, and the tracing of absorption and movement of salts by means of radio-active isotopes. Professor Hoagland is author of many scientific articles and of the recently published book entitled "Lectures on the Inorganic Nutrition of Plants."

Through his marked achievements in science, Professor Hoagland has earned many well deserved honors. He is a member of the National Academy of Sciences, and has served as president of the American Society of Plant Physiologists, of the Western Society of Soil Science, of the Western Society of Naturalists, and of the Pacific Division of the American Association for the Advancement of Science. He was the recipient of the first Stephen Hales Prize Award of the American Society of Plant Physiologists in 1929, and was joint recipient of the American Association Prize in 1940.

Professor Dennis Robert Hoagland, past-president of our Society, has been selected as the recipient this year of the Charles Reid Barnes Life Membership, in recognition of his researches on the mineral nutrition of plants. In behalf of the American Society of Plant Physiologists, the committee expresses its appreciation of Professor Hoagland's outstanding contributions to the plant sciences, and it extends to him best wishes for continued success in his field of scientific investigation.

The New England Section.—The New England Section of the American Society of Plant Physiologists will resume its schedule of annual meetings

May 17–18 at Harvard University, Cambridge, Massachusetts. Dr. Kenneth V. Thimann, Harvard University, is Chairman and Dr. Linus H. Jones, Massachusetts State College, is Secretary. The meeting, which will be held in the Harvard Biological Laboratories beginning on Friday afternoon, is open to all interested in the subject of plant physiology.

E. C. Miller.—The American Society of Plant Physiologists conveys its felicitations to DR. MILLER, on the occasion of his retirement after 35 years of active service in teaching and research. In recognition of his distinguished career as a scientist, the society presents a brief biographical record of DR. MILLER and his professional work.

DR. EDWIN CYRUS MILLER was born December 16, 1878, in a log cabin on a farm near Baltimore, Ohio. He received the B.S. degree in 1904 and the A.B. degree in 1906 from the old National Normal University at Lebanon, Ohio. Then followed an A.B. and the Ph.D. from Yale University in 1907 and 1910, respectively.

In the fall of 1910, DR. MILLER came to Kansas State College, where he was successively Instructor, Assistant Professor, Associate Professor, and, since 1919, Professor of Plant Physiology. He became a member of the Kansas Agricultural Experiment Station Staff in 1911 and has been Plant Physiologist of that Station since 1919. DR. MILLER served two years as acting head of the Department of Botany and Plant Pathology.

DR. MILLER continued to be active in the teaching of plant science at Kansas State College throughout his career, lecturing to students in the introductory course of plant science until two years before retirement and in the specialized courses in Plant Physiology until one year before retirement. One of his outstanding contributions to the teaching of plant science at Kansas State was his use of plants having economic value, plants for the most part known by the student, to illustrate his lectures and writings. He maintained an interest in the welfare of students and they were welcome to his counsel at all times. This interest in students was further manifested by the conscientious manner in which he carried out his assignment as a member of the College Committee dealing with failing students, an assignment which he held the last 15 of his 35 years at Kansas State College. His book, "Plant Physiology," which appeared in 1931 and the revised edition in 1938 has provided an outstanding text with extensive bibliographies for advanced classes, the graduate student, and the research worker.

DR. MILLER'S research has had to do largely with the water relations and chemistry of plant life. His Ph.D. problem was a basic study of the changes and processes involved in the germination of a fatty seed (that of *Helianthus annuus*) which DR. WILLIAM CROCKER said in a review in the Botanical Gazette showed "both chemical and biological excellence." His first work at Kansas State College was the completing of two studies initiated during the progress of his doctorial problem at Yale. One had to do with the changes in the oily reserve of this seed at different stages in the development of the seedling, and the other with the origin of chloroplasts in its cotyledons.

DR. MILLER'S first studies as a member of the Experiment Station staff were conducted at the Garden City Branch Station, and were concerned with the root systems of crop plants. They were technical studies with practical applications. They included a comparative study of the extent of root systems and leaf areas of corn and the sorghums, the relative water requirements of these plants, the daily variation of water and dry matter in their leaves, and their relative transpiration. These studies showed that the sorghum root system was better adapted to get water from the soil and while the sorghums lose more water per unit area of leaf surface, their total leaf surface was less than that of corn; thus is explained the observation of the farmer that sorghums withstand drought better than does corn. Then followed a study on the daily variation of the carbohydrates in the leaves of corn and the sorghums, and three studies in which DR. MILLER shared authorship: one having to do with the temperatures of leaves of crop plants; the second with the transpirational loss and water requirement of certain weeds and crop plants of Kansas; and the third, which has been widely cited, with the elemental composition of the corn plant. DR. MILLER'S later station work was concerned with wheat of which his technical work on, "A Physiological Study of the Winter Wheat Plant at Different Stages of Its Development" was the most extensive. Two studies in which DR. MILLER was joint author were concerned with the relation of leaf rust infection to (a) yield, growth, and water economy and (b) diurnal transpiration in wheat. Three later studies on wheat conducted jointly with graduate students were concerned with (a) the influence of awns upon the rate of transpiration, (b) a consideration of the morphological nature and physiological functions of the awn, and (c) the effect of defoliation on the functions of winter wheat. DR. MILLER was author or co-author of 24 technical publications.

DR. MILLER served as President of the American Society of Plant Physiologists and as a member of the Editorial Board of that society. He is a member of Sigma Xi, Phi Beta Kappa, Phi Kappa Phi, A.A.A.S. (Fellow), Alpha Zeta, Gamma Signa Delta, and Farm House Fraternity.

DR. MILLER has several hobbies from which he has derived diversion from scientific endeavors. One is a survey of the life and work of ABRAHAM LINCOLN which has resulted in a manuscript for a book entitled, "The Rise of Abraham Lincoln," a factual study of the life of this great American as seen through the eyes of a scientist. The second is a consideration of the importance of the corn plant in the life of man which has also resulted in a manuscript of book length. Both manuscripts are now being considered for publication. One of DR. MILLER'S chief hobbies, aside from writing, was playing golf. His golf game, which he played for the fellowship involved rather than proficiency at the game, was well described in his droll statement, "I play brilliantly at times."

To know DR. MILLER intimately is to appreciate his many virtues which include straight-forwardness, a sense of fair play, a love of humor, friendliness, and high integrity. With the title Professor Emeritus of Plant Physi-

ology, DR. MILLER has retired to the farm on which he was born and which he now owns. Through his book on Plant Physiology he will continue to serve plant physiologists throughout the world. Almost one-fourth of the 6,000 copies sold to date (1945) have gone to students and libraries in foreign countries.

National Research Council Representative.—PROFESSOR B. S. MEYER of Ohio State University has been appointed representative of the American Society of Plant Physiologists on the National Research Council.

Editorial Board of Plant Physiology.—DR. F. P. CULLINAN of the Bureau of Plant Industry of the U. S. Department of Agriculture, Beltsville, Maryland, has been elected to a three year term on the Editorial Board of Plant Physiology by the Executive Committee of the American Society of Plant Physiologists.

Biological Field Stations of the World.—HOMER A. JACK. No. 1, Vol. 9, of Chronica Botanica. Chronica Botanica Co., Waltham, Massachusetts, and G. E. Stechert and Co., New York City. 73 pages. $2.50.

A valuable addition to the Chronica Botanica collection, this booklet represents not only a coordination of the subject material existing in the literature, but also first-hand information on 79 biological stations in 18 countries which were visited by the author.

After a brief historical account of the location, administration, and functions of the various biological stations, they are generally described and compared. The second part of the book is organized as a directory of the Biological Stations arranged conveniently by countries. As much direct information as possible is included with each station, no matter how obscure; but of necessity, the descriptive accounts are corrected to 1940—before World War II became wide-spread. Several wood-cuts of biological stations are included.

Organic Preparations.—CONRAD WEYGAND. Interscience Publishers, Inc., 215 Fourth Avenue, New York 3, N. Y. 534 pages. $6.00.

Available for the first time is this capable translation of Part II "Reaktionin" of WEYGAND'S Organisch-chemische Experimentierkunst, published in Leipsig in 1938. Since all organic reactions consist of the breaking of existing bonds or the formation of new ones, the author has advantageously organized the chapters so that each is the discussion of the formation or cleavage of such bonds. The book advances from the simpler linkages to the more complex, and chapters are subdivided into the various reactions. A theoretical discussion under each heading precedes the laboratory instructions so that essentially the book combines the best features of a text and a laboratory manual for organic preparations.

The standard methods for preparations are footnoted extensively with references from the chemical literature. An extensive subject index is included in this fine, modern book.